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**Mapping domains of gene expression in
the Malpighian tubule of *Drosophila
melanogaster* by enhancer trapping**

by

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**A thesis submitted to the University of Glasgow for the
degree of Doctor of Philosophy**

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Author's Declaration

The study described here has not been presented for any other qualification or degree. All work was carried out by the author except the generation of the enhancer trap lines, which was carried out together with Dr. J. D. Armstrong and Dr. M. Y. Yang within the framework of collaboration, and where otherwise stated.

M. A. Sözen

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Abbreviations

<i>β</i> -gal	<i>β</i> --galactosidase
GFP	Green Fluorescent Protein
PCR:	Polymerase Chain reaction
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
<i>Kr</i>	Krüppel
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
V-ATPase	Vacuolar adenosine triphosphatase
DH	Diuretic hormone
MIP	Major intrinsic protein
BLIP	Basolateral intrinsic protein
PBS	Phosphate buffered saline
UASG	Upstream activating sequence for galactose
<i>Dr</i>	<i>Drop</i>
IR	Inverted repeat
CyO	Curly of Oster
DAB	Diaminobenzidine
NBT	Nitrobluetetrazolium
EDTA	Ethylenediamine tetraacetic acid
DMSO	Dimethyl sulfoxide
KOAc	Potassium acetate

DDT	Dithiothreitol
SDS	Sodium dodecyl sulfate
TBE	Tris-borate
TRIS	Tris(hydroxymethyl)aminomethane
HRP	Horse radish peroxidase
IPTG	Isopropylthio- β -D-galactoside
5-HT	5-hydroxytryptamine
MT	Malpighian tubules
AMT	Anterior Malpighian tubules
PMT	Posterior Malpighian tubules

SUMMARY

The Malpighian tubule of *Drosophila melanogaster* is a valuable epithelial model for developmental, physiological and genetic studies. A set of 700 P{GAL4} enhancer-trap lines of *D. melanogaster* was screened for patterned β -galactosidase reporter gene expression in the Malpighian tubules. Of these, around 20% show some internal patterning within tubules, and 1% appeared to be specific to tubule cell subpopulations.

Staining patterns were compared and used to chart the patterns of gene expression in the tubule. By counterstaining nuclei with ethidium bromide, it proved possible to assemble a numerical map of the cell types in each tubule subdomain. It was found that tubules could be subdivided into at least five sub-domains and multiple cell types defined by gene expression. Remarkably, the patterns of gene expression and the numbers of both principal and secondary ("stellate") cell types within each domain, are reproducible to single-cell precision between individual animals.

The numbers of cells, both in the whole tubule and in individual compartments, are practically invariant both between individuals of a particular line and between lines. Comparison with the regions previously identified by morphological or physiological techniques, revealed that a genetic boundary can always be found which corresponds with the known division. In addition, several new subdomains are proposed from enhancer trap analysis alone; additional experiments confirm the physiological significance of the proposed subdivisions.

Previous studies had identified two tubule cell types: "principal" (type I) and secondary "stellate" (type II) cells in tubules. In this study, it was found that each of these two cell types can be subdivided further, and that there is a previously unsuspected heterogeneity among morphologically indistinguishable principal cells. In addition, a previously unreported neuroendocrine cell type was identified, which reacts for the HRP insect neuronal marker.

Using transport or expression assays, domains of physiological function have also been mapped. Invariably they respect the boundaries defined by enhancer activity. These genetic compartments can also be visualised *in*

vivo, both in transgenic and wild-type flies, providing an "identified cell" system for epithelial physiology. Building upon recent advances in *Drosophila* Malpighian tubule physiology (Dow et al., 1994a; Dow et al 1994b) the present study confirms this tissue as a singular model for integrative physiology.

A preliminary developmental analysis of the expression patterns reported by these lines provides the first evidence that both stellate and principal cells are specified and distinct as early as the late embryo. Similarly, the principal cell lines have been useful in a study of temperature effects on variegation in tubules. By cloning and characterising new candidate tubule-specific genes, identified by enhancer trap lines on the basis of their expression patterns, both novel transcription units and novel regulatory mechanisms may be uncovered.

This is the first epithelium to be subjected to such a genetic analysis, and these results confirm the utility of enhancer trapping as a means of identifying genetic boundaries which can subsequently be shown to correspond with functional tissue domains.

Chapter 1: Introduction

1.1. *D. melanogaster* as an important experimental organism

The fruit fly *Drosophila melanogaster* has long been used as an experimental organism in genetical studies. In particular, a remarkable expansion in the definition of the *Drosophila* genome has been witnessed over the last two decades. *Drosophila* has emerged as an organism of choice for molecular genetic investigations of eukaryotic biology and with the contributions of many talented workers attracted to this field, the rapid advances in molecular technology have been achieved. These advances have both provided new and sophisticated tools and generated novel insights. (Lindsey and Zimm, 1992). As a result, *D. melanogaster* with its intermediate level of genomic complexity, in combination with its sophisticated genetics, has turned out to be particularly well suited to study the basic problems in metazoan biology (Rubin, 1988).

Before the advent of molecular biology, genes were identified exclusively through the existence of mutant alleles. Advances in amino-acid and nucleotide sequencing, polymerase chain reaction (PCR), cloning of DNA sequences, transposable elements, hybrid dysgenesis and transformation, have led to a shift in emphasis to the normal structure and function of genes. Many new genes were discovered through the ability to identify a gene from either its protein product or the homologous product from another species. In addition, enhancer trap analysis has extended these discoveries further with interesting expression patterns over the last decade (Lindsey and Zimm, 1992).

D. melanogaster is now widely used, particularly in many new biochemical, cell biological, and physiological techniques, to research problems requiring a multidisciplinary approach as well as classical and molecular genetics (Rubin, 1988). As an example, the dissection of fluid secretion, ion transport mechanisms and signal transduction pathways in the Malpighian tubules also requires a multidisciplinary approach. This model organism is very well-suited for this purpose.

Classically, insect Malpighian tubules have long been used as models of epithelial fluid secretion and its neurohormonal control (Maddrell, 1991; Maddrell and O'Donnell, 1992). However, none of the species studied to

date are particularly suited to molecular genetic analysis. Potentially, the only organisms available with the requisite genetic tools are *D. melanogaster*, mouse and perhaps zebra fish. Recently, it has been shown that the *Drosophila* renal tubule, although the smallest ever studied, is nonetheless amenable to physiological analysis. Indeed, it possesses a remarkable repertoire of transport, pharmacological and molecular properties (Dow, 1994).

The Malpighian tubule of *D. melanogaster* has recently emerged as a novel phenotype for the extension of studies of ion transport beyond that revealed by the techniques of classical physiology. To obtain further information, it is essential to adopt a molecular genetic approach in which the relevant genes are identified, characterised and mutated. The Malpighian tubules of *D. melanogaster* seem to be the best candidate to realise these tasks (Dow, 1994).

It is also well suited for studies of very complex gene regulation mechanisms, either that control spatial or temporal patterns of expression only in one specific cell type, or in several tissues, or at several different times during development (or both); and in particular, of their regulatory DNA sequences (Maniatis *et al*, 1987).

1.2. General introduction to the Malpighian tubule of *Drosophila melanogaster*

The Malpighian tubules are excretory and osmoregulatory organs, analogous to the vertebrate kidney, which excrete nitrogenous wastes and excess water and regulates osmotic balance. They have also a great importance as a transporting epithelial model due to the molecular, physiological and pharmacological analysis that can be applied to them.

The Malpighian (renal) tubule is also a model system for cell fate specification in development. The generation of tubules from ectodermal anlagen has been shown to be directed by neurally-derived tip cells involving the normal expression of the segment polarity gene, *wingless* during the period of cell proliferation (Skaer, 1989 and Skaer and Martinez-Arias, 1992). The products of the genes *cut*, *caudal*, *Krüppel*, *crumbs* and *stardust* have been implicated in tubule development (Gaul and Weigel, 1990; Liu and Jack, 1992; Skaer and Martinez-Arias, 1992; Tepass and Knust, 1990; Grawe *et al.*, 1996). However, nothing is known

about the genes involved in the specification and differentiation of cell types (Hoch et al., 1994; Skaer, 1993).

Morphologically, the tubules have been described by Wessing (Wessing and Eichelberg, 1978) **Figure 1.1**. The structure and function of the tubule cells are very different between the proximal and distal ends (Bradley, 1984; Wessing and Eichelberg, 1978; Skaer, 1993). Four different domains (at the anterior only) and two different cell types have been identified in the Malpighian tubules.

Recently, it has been shown that the tubules of this very small insect are amenable to experimental study. The tubules themselves are easily dissected and manipulated and their activity can be simply measured (Dow *et al.*, 1992; Dow *et al.*, 1994a). A full repertoire of transport and pharmacological properties can be assigned to this model epithelium. (Dow and Maddrell, 1993; Dow *et al.*, 1994a; Dow *et al.*, 1994b; O'Donnell *et al.*, 1996). In addition to the physiological merits of the Malpighian tubules, they also provide an ideal model for molecular dissection of signal transduction. This epithelial model is first to be studied in a molecular genetic context.

1.3. Morphological Characteristics of Malpighian tubules

1.3.1. Basic structure

The Malpighian tubules are excretory organs, functionally analogous to the vertebrate kidney. They are long, blindly-ending tubes that empty into the gut at the junction of the hindgut and posterior midgut. There are a total of four tubules, 2 oriented anteriorly (anterior Malpighian tubules or AMT) and 2 oriented posteriorly (posterior Malpighian tubules or PMT). The tubules are coiled into the body space and join up as pairs before connecting into the gut through a short ureter (**Figure 1.1 a**). The terminal regions of the initial segments of the anterior tubules are embedded in fat body, and the tips of the posterior tubules become attached to the posterior part of the hindgut in the abdomen. These attachments are thought to be mediated by connective tissue cells (Wessing and Eichelberg, 1978).

Classically, in adults the posterior tubule pair is described as being clearly longer than the anterior one (approx. 25 %), and the anterior tubules are

shorter than those of the larva (Wessing and Eichelberg, 1978). However, a more recent report suggests that adult anterior tubules are longer than the posterior ones (Janning *et al.*, 1986).

Three tubule subregions can be defined by electron microscopy; initial segment, transitional segment, and main segment. Additionally, two cell types (Type I and II) were identified. Electron micrographs reveal striking morphological differences in the tubules between the anterior and posterior ends, reflecting these functional differences. (Wessing and Eichelberg, 1978; Skaer, 1993). Morphologically, the tubules have been described in detail by Wessing (Figure 1.1 b).

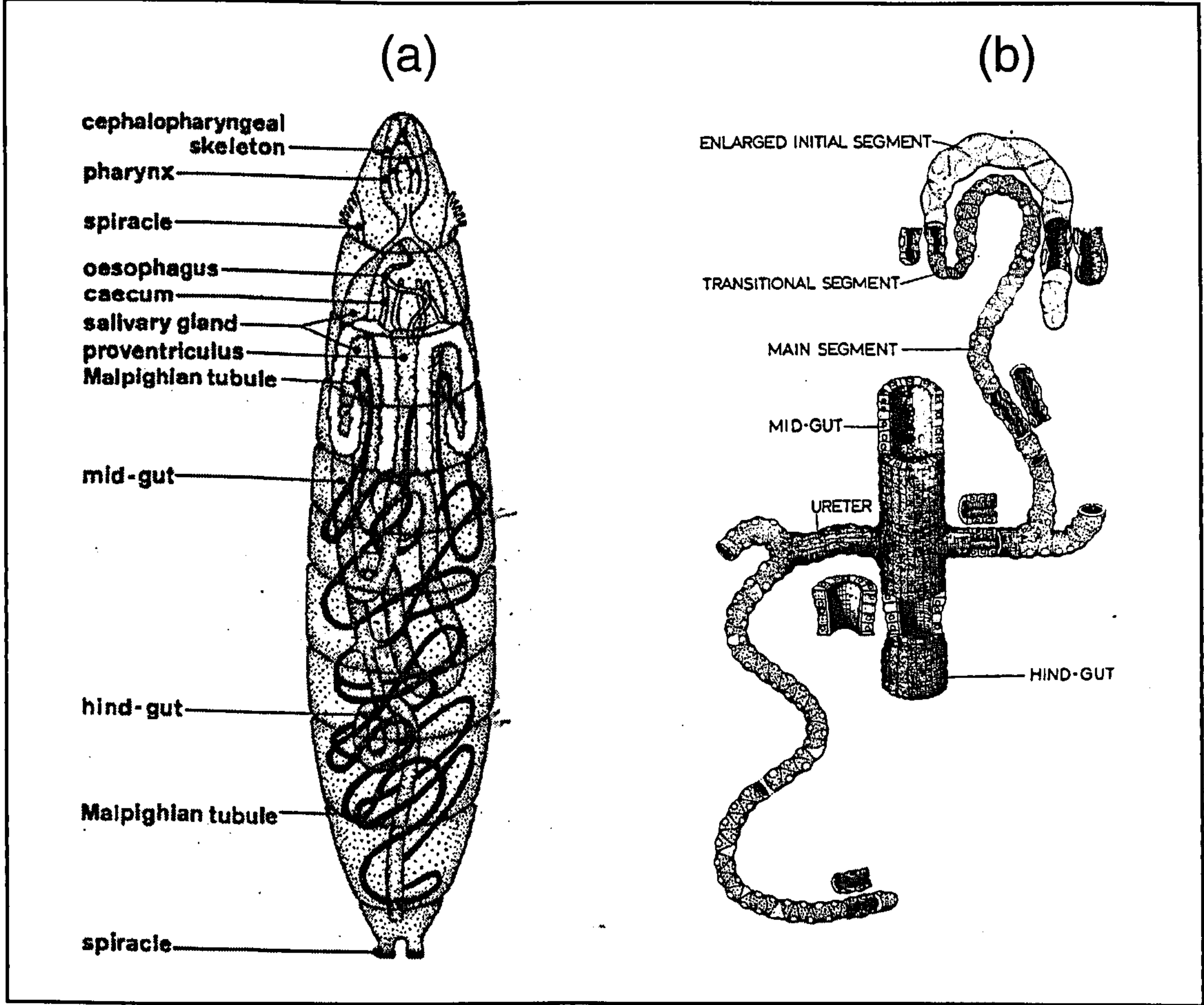


Figure 1.1. Classical morphological analysis of tubule structure.

a) Larval tubules showing positioning within body cavity.

b) Detail of tubule regions and morphology. Adult tubules (not shown) are classically considered to be similar (Reproduced from Wessing and Eichelberg 1978).

1.3.2. The subregions of Malpighian tubules

Classically, tubules have been studied morphologically, physiologically and ultrastructurally. Much of the work in this thesis will refine this classical map, and so it is worthwhile to describe it in some detail here.

The previous techniques suggest that the anterior pair of larval tubules is divided into three different segments: a dilated initial segment (distal), a long main segment ending in the common ureter proximally, a short bent transitional segment joining these two regions together. The tubules are connected to the gut by a short ureter. Transitions between these segments are considered to be gradual. By contrast, the posterior tubules are thought to comprise solely a main segment running into the common ureter in the larvae and pupa (Wessing and Eichelberg, 1978).

Adult tubules are thought to be similar to those of the larvae with a few exceptions. Anterior tubules no longer possess an enlarged initial segment although the cells of this region have microvilli lacking central channels of endoplasmic reticulum. These cells are followed by those which possess structures associated with the transitional segment of the larvae (formation of concretions in intermicrovillar spaces) (Wessing and Eichelberg, 1978).

Ultrastructurally, the organization of the ureter cells was described as being different in the distal and proximal regions. The distal cells were found to be similar to the cells of the main segment, but vary in some ways. The proximal section of the ureter had a structure similar to that of the midgut (Wessing and Eichelberg, 1978).

As a summary, the ultrastructural studies suggest a subdivision into four regions, namely an initial (dilated in larvae), non-transporting part unique to the anterior pair of tubules, a main segment which produces the primary urine, a transitional segment and a short ureter (Wessing and Eichelberg, 1978). The transitional segment might be considered to be merely an area in which the properties of initial and main segments merge seamlessly.

In addition, it had been reported previously (Wessing and Eichelberg, 1978) that the initial segments of larval anterior tubules were lost during metamorphosis and the anterior tubules of *D. melanogaster* have a

prominent dilated initial segment with white material, presumably the concretion bodies reported in larvae (Zierold and Wessing, 1990) in the lumen. This was opposed by Janning *et al.* (1986).

1.3.3. The cell types of Malpighian tubules

Previous reports identified two major cell types in tubules (Wessing and Eichelberg, 1978): a principal, type I cell and a rarer type II or “stellate” cell. The principal cell is a classic squamous epithelial cell with microvillate apical border and involuted basal border, whereas the minor stellate cell shows a cruciform morphology which appears to “slot in” along the interstices between principal cells. There is also a clear dichotomy between the structure of the type I cells in the initial segment and those of the rest of the tubule, and between the morphologies of the principal and the type II cells in the mentioned regions (Wessing and Eichelberg, 1978).

Type II cells of the initial segment were described as being extremely flattened and thinner than the basement lamina (about 50 nm). These cells were shown to contain few structures, seldom possess basal infoldings, and possess neither microvilli nor pinocytotic vesicles (Wessing and Eichelberg, 1978)

Type I cells of the initial segment were described as being wider in comparison with the type II cells of this segment but relatively narrow compared with the larger type I cells of the main segment. They have numerous basal infoldings, but only a few microvilli lacking central canals.

The structure of the cells of the initial segment were reported to vary, either having a dense cytoplasm (dehydrated) and with wide lamina of basal infoldings of the plasmalemma; or loose (hydrated), and with narrow lamina of the infoldings. The infoldings are few in number. A number of transitional states exist between the dehydrated-appearing and the hydrated-appearing cells. The different appearance of these cells is argued not to be due to different kinds of cells, but to different phases in the functioning of a single cell type (Wessing and Eichelberg, 1978).

The principal cells (type I) in the main segment are large epithelial cells joined by septate junctions and have basal infoldings and apical

microvilli. The mitochondria are mainly in the basal and the apical regions of these cells associated with active transport. Interestingly, mosaicism among principal cells has been described in position-effect variegated mutants for the *Drosophila white* locus (Van Breugel, 1973; Clancy, 1955; Hartman-Goldstein et al., 1976); however the significance of this not clear.

Besides type I cells in the main segment, a few smaller, flatter and lighter cells (type II) were described. They have shorter microvilli but no storage vacuoles in them (Wessing and Eichelberg, 1978).

The cellular organization of the transitional segment was described as largely corresponding to that of the main segment except for the fact that spaces, separated from the lumen, arise from lateral conjugations of the microvilli (Wessing and Eichelberg, 1978).

A combination of painstaking developmental observation and genetic markers has also identified the presence of a neuroectodermal tip cell at the end of each developing tubule, and shown that cells with similar properties persist into larval life (Hoch *et al.*, 1994).

1.3.4. Embryology and Developmental Changes

The Malpighian tubules develop from ectodermal anlagen through the processes of the specification of primordial cells, their proliferation, rearrangement and final cell differentiation. These processes are coordinated by intercellular communication (Skaer and Martinez-Arias, 1992). During embryogenesis, the tubules emerge as four outpushings from the proctodeum (hindgut infolding), and grow by cell division initially and then by rearrangement. In the end, they result in a long, tubular, single-cell-layered epithelium. In addition, the genetic circuitry charted by Skaer (1993) underlying the segregation of Malpighian tubules from proctodeum can be described as follows:

The embryonic termini are specified through a signalling cascade by maternally supplied products. As a result of this specification, the expression of *tailless* and *huckebein* is switched on in these regions. These two zygotic terminal genes acting through the transcription factor, *fork head* (Weigel *et al.*, 1989), initiates the expression of *Krüppel* (*Kr*) in the posterior domain (Gaul and Weigel, 1990), which forms the

proctodeum after gastrulation. *Kruppel* is a segmentation gene of gap class, which encodes a zinc finger DNA binding protein (Rosenberg *et al.*, 1986). The *Kr* expression, showing a dynamic pattern of expression in the proctodeum, gradually becomes restricted to a ring of cells, which push out to form the tubule primordia (Gaul *et al.*, 1987). This process can not be observed in *Kr* mutant embryos, suggesting *Kr*'s critical role in the specification of tubule primordial cells. The differentiation of the Malpighian tubules from the gut may be controlled, at least in part, by *Kruppel*, and *cut* genes (Blochlinger *et al.*, 1988; Liu and Jack, 1992). Mutations of both *Kruppel* (Harbecke and Janning, 1989) and *cut* (Liu *et al.*, 1991) cause a transformation of Malpighian tubule cells to cells that form an enlargement of the gut at the midgut-hindgut junction.

The subsequent cell proliferation in each primordia has been shown to be directed by neurally-derived tip cells following their selection (Skaer, 1989). This is achieved by a mechanism regulating cell division during the organogenesis of the Malpighian tubules in *D.melanogaster*. Following the segregation of the tubules from hindgut, a "tip cell" becomes apparent at the distal end of each growing primordium, showing a distinct character both in its position and its behaviour. This specialised cell does not divide as other cells in the primordium are proliferating. The ablation of the tip cells results in arrest of mitosis prematurely in the remaining cells of the tubule of which it is a part. The tip cells are essential for the normal pattern of cell division in the development of the tubules. Cell division also depends upon the normal expression of the segment polarity gene, *wingless*. *Wingless* is also required for normal eversion of the four primordia to form tubules (Skaer and Martinez-Arias, 1992).

Each tip cell emerges from a cluster of equipotent cells, by division of a tip mother cell in each tubule primordium through a series of inhibitory cell interactions (Hoch *et al.*, 1994). The selection of each tip cell from each tubule primordium has been shown to involve lateral inhibition. This process closely resembles neurogenesis and it is mediated by proneural and neurogenic genes (**Figure 1.2**). At that stage, each tip cell differentiates to express neural characteristics, adopting a second fate, besides the mitogenic role during embryogenesis (Hoch *et al.*, 1994; Wilkins *et al.*, 1995).

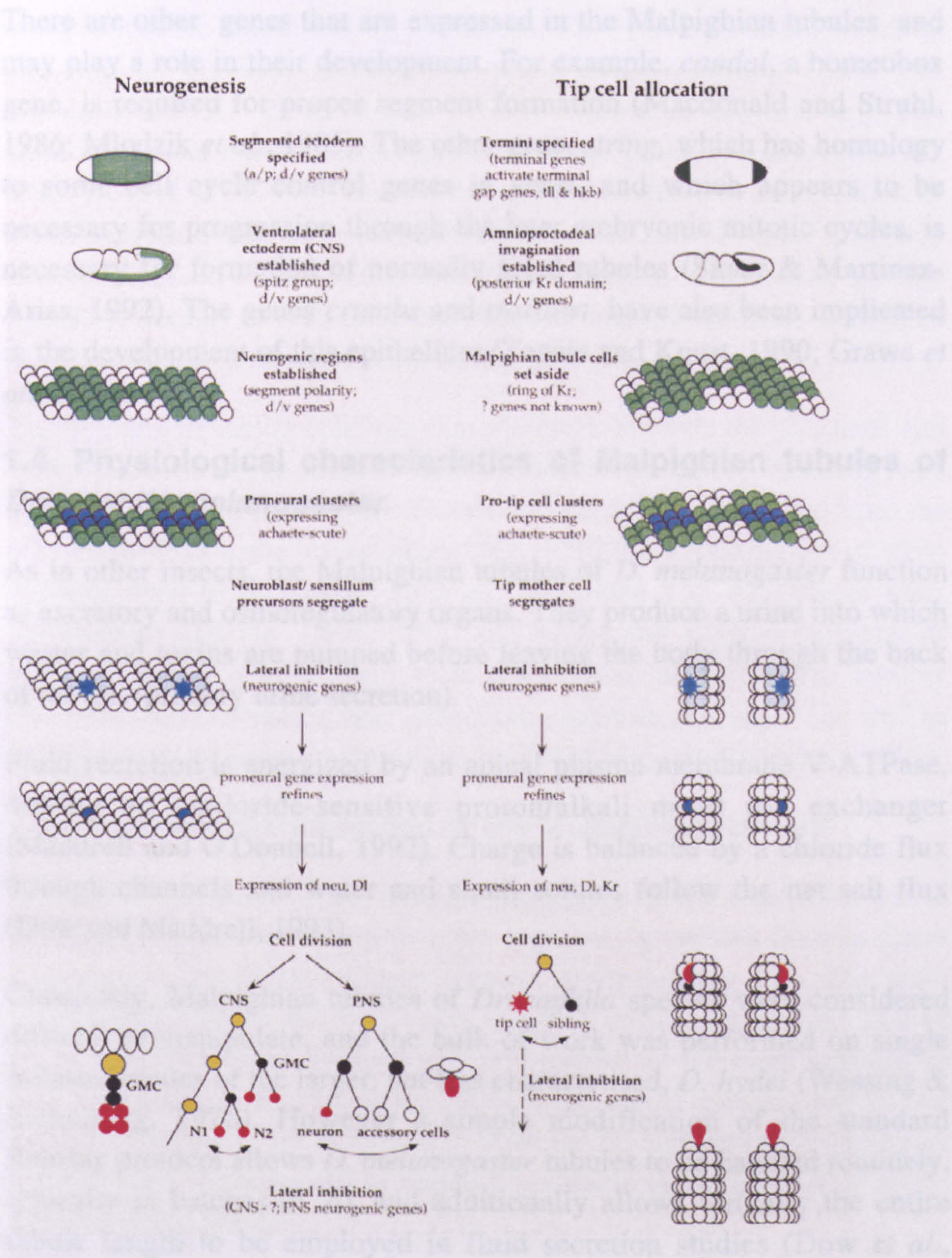


Figure 1.2. The sequence of events underlying neurogenesis and tip cell allocation.(reproduced from Hoch *et al.*, 1994)

There are other genes that are expressed in the Malpighian tubules and may play a role in their development. For example, *caudal*, a homeobox gene, is required for proper segment formation (Macdonald and Struhl, 1986; Mlodzik *et al.*, 1985). The other gene, *string*, which has homology to some cell cycle control genes in yeast, and which appears to be necessary for progression through the later embryonic mitotic cycles, is necessary for formation of normally-sized tubules (Skaer & Martinez-Arias, 1992). The genes *crumbs* and *stardust* have also been implicated in the development of this epithelium (Tepass and Knust, 1990; Grawe *et al.*, 1996).

1.4. Physiological characteristics of Malpighian tubules of *Drosophila melanogaster*

As in other insects, the Malpighian tubules of *D. melanogaster* function as excretory and osmoregulatory organs. They produce a urine into which wastes and toxins are pumped before leaving the body through the back of the gut (primary urine secretion).

Fluid secretion is energized by an apical plasma membrane V-ATPase, driving an amiloride-sensitive proton/alkali metal ion exchanger (Maddrell and O'Donnell, 1992). Charge is balanced by a chloride flux through channels and water and small solutes follow the net salt flux (Dow and Maddrell, 1993).

Classically, Malpighian tubules of *Drosophila* species were considered difficult to manipulate, and the bulk of work was performed on single isolated tubules of the larger, but less characterised, *D. hydei* (Wessing & Eichelberg, 1978). However a simple modification of the standard Ramsay protocol allows *D. melanogaster* tubules to be handled routinely, typically in batches of 20, and additionally allows virtually the entire tubule length to be employed in fluid secretion studies (Dow *et al.*, 1994a). Epithelial transport mechanisms (summarised below) identified in the insect's tubules are comparable with those in tubules of other insect species which means that a complete physiological and molecular dissection of this tissue seems to be possible that may not be feasible in any other transporting epithelium (Dow *et al.*, 1994b).

Tubules also display a full repertoire of transport and pharmacological properties. In particular, a plasma-membrane V-ATPase energised fluid

transport can be stimulated by cAMP and Ca^{2+} ; or by cGMP, which in turn can be elevated through the nitric oxide pathway (Dow and Maddrell, 1993; Dow *et al.*, 1994a; Dow *et al.*, 1994b). The identification of extracellular ligands for these pathways (Davies *et al.*, 1995; Dow *et al.*, 1994b), and the demonstration that control of cation or anion secretion can be separately assigned to one or other pathway (O'Donnell *et al.*, 1996), further increases the utility of the model for cell-signalling studies as well as a role of major intrinsic proteins (MIPs) in fluid generation (Dow *et al.*, 1995).

The function of tubule cells are very different between the proximal and distal ends in most insects studied (Bradley, 1984; Wessing and Eichelberg, 1978). Among them, active reabsorption of glucose from the primary urine of some insects (Knowles, 1975) and accumulation of some cationic dyes from lumen such as rhodamine 123 by stellate cells (Meulemans and De Loof, 1992) and active and passive transport of various organic solutes (uric acid, acylamides, alkaloids and cardiac glycosides) and inorganic ions (phosphate, sulphate and Mg^{2+}) as well as ions and fluid have been reported so far (Maddrell and O'Donnell, 1992).

1.4.1. Functional subregions of the Malpighian tubules

The initial segments of the anterior tubules have been reported not to secrete any detectable fluid; the main segments generated fluid in direct proportion to the length of main segment in the bathing drop. The main segment starts below the transitional segment, and runs to around 35% of the entire length from the upstream end (distal) of the main segment to ureter (**Figure 1.3.**). This lower 35% of the tubule (approximately one third) was reabsorptive in function; however no classical morphological boundary can be observed to match this functional division (O'Donnell and Maddrell, 1995).

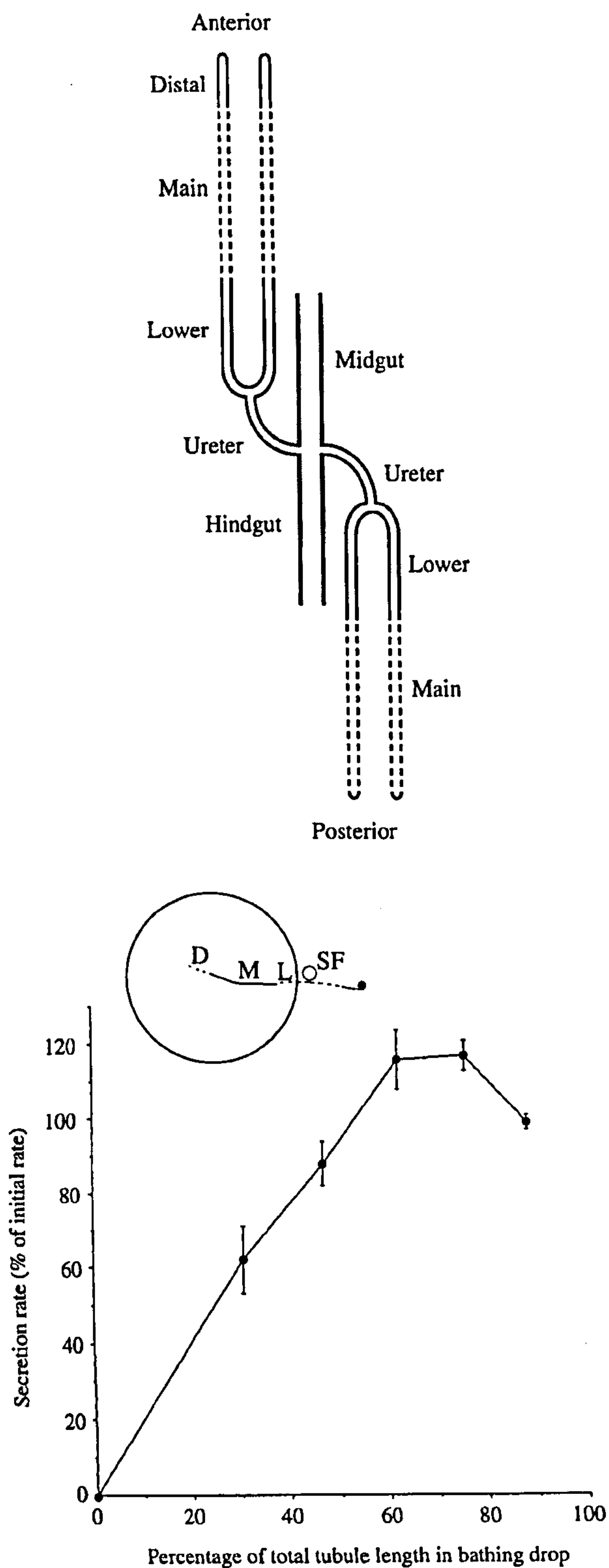


Figure 1.3. Functional subregions in the Malpighian tubules

(a) Schematic diagram of the arrangement of the functional regions of the Malpighian tubules. **(b)** Changes in relative rates of fluid secretion by Malpighian tubules when successive lengths were pulled out from the bathing drop (reproduced from O'Donnell and Maddrell, 1995). Abbreviations: D: distal, M: main, L: lower regions, SF: secreted fluid

1.4.2. The functions of the cell types of the Malpighian tubules

Based mainly on ultrastructural data, type I cells of the initial segment are thought to transport substances in various ways (Wessing and Eichelberg, 1978). One of these methods is pinocytosis: the basal plasmalemma forms vesicles, which detach from the basal infoldings, and release their contents into the lumen. Other pathways of transepithelial passage of substances in the cells of the initial segment of the Malpighian tubules of larval *D. melanogaster* proposed by Wessing and Eichelberg (1978) are: 1. Accumulation of penetrated substances in the cisternae of the endoplasmic reticulum. 2. Transport through the cytoplasm of substances not bound to membranes. 3. Free transport through the cytoplasm along the outer plasmalemma. 4. Passage through the inter-cellular space. 5. Passage through the intercellular space after passing the cell. However, the cells of initial segment do not secrete uric acid.

The main segment cells, contrary to those of the initial and transitional segment, are argued to mainly resorb despite a lack of physiological proof (Wessing and Eichelberg, 1978). The cells of this segment are described as secreting uric acid. Under experimental conditions they are also capable of transporting the injected substances from the haemolymph into the lumen. The transport mechanism is similar to that of the initial segment cells, but varies with the complexity of the system of basal cell infoldings. Besides having a transporting function ions can be stored in the endoplasmic reticulum and its vacuols for longer periods of time.

More recent data have suggested that the stellate cells may act as chloride-transporting cells (O'Donnell *et al.*, 1996; Pannabecker *et al.*, 1993), or to reabsorb solutes from the urine (Meulemans and De Loof, 1992). It was found that they stain immunocytochemically for BLIP, a member of the MIP family of proteins which include water channels (Dow *et al.*, 1995).

1.4.3. Ion transport in the Malpighian tubules of *D. melanogaster*

The Malpighian tubules of insects are excretory organs made of a single layer of epithelial cells. They are worthy of study due to their remarkably high transport rates of both water and ions. The cells of the blood sucking insect *Rhodnius prolixus* for instance can transport their own weight of

fluid every 15 seconds (Maddrell, 1991). *Drosophila* tubules achieve an even higher rate of transport on a per-cell basis, making them probably fastest known secretory cells (Dow *et al.*, 1994a).

As well as ions and fluid the tubules also transport inorganic ions (**Figure 1.4**) such as phosphate, sulphate and Mg^{2+} ; organic compounds such as uric acid ; and low mw solutes, possibly through the cell-cell septate junctions (Maddrell and O' Donnell, 1992).

Until recently this process was thought to be understood in *Rhodnius prolixus*. A cation pump was thought to pump sodium or potassium or both from the cells into the lumen energising the system, while chloride moved down its electrochemical gradient into the lumen through chloride channels. This was accompanied by passive transport of sodium, chloride and potassium from the haemolymph into the cells (**Figure 1.5 a**). New evidence however implicates the V-ATPase as having a critical function. This is seen by treating the cells with either NEM or Bafilomycin (V-ATPase inhibitors) both of which lead to a drop in transport (Bertram *et al.*, 1991). Also it has been shown that anti V-ATPase antibodies bind to the luminal face of the *Manduca sexta* Malpighian tubule plasma membrane (Russell *et al.*, 1992). It has been proposed therefore that the V-ATPase in conjunction with a cation/ H^+ antiporter acts as the driving force rather than a sodium/ potassium pump (**Figure 1.5 b**). The activity of the tubules in *Rhodnius* is controlled both by (DH) diuretic hormone and 5-HT synergistically (Maddrell *et al.* , 1991; Maddrell *et al.* , 1993).

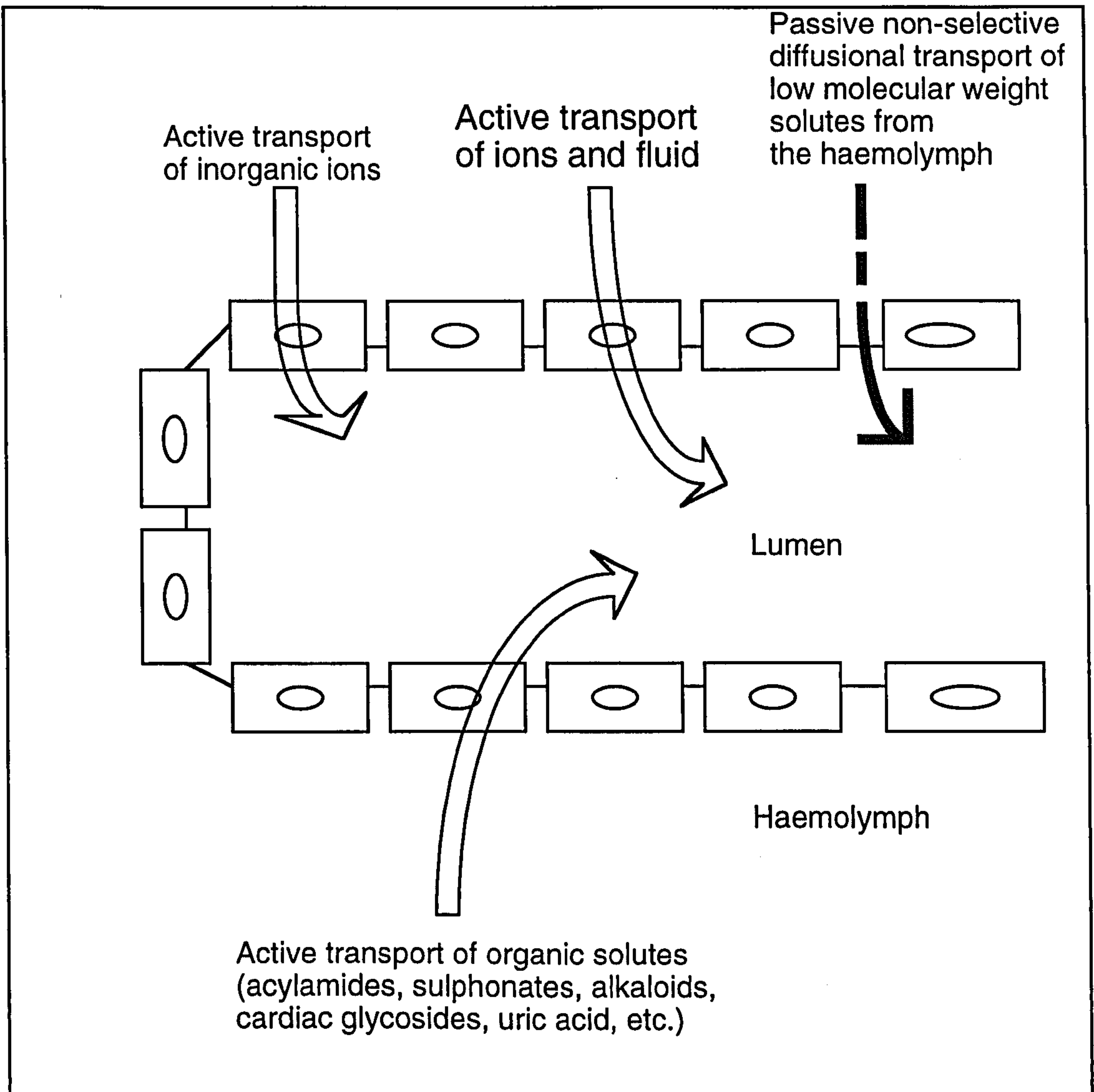


Figure 1.4. Physiological involvements of the Malpighian tubules. Malpighian tubules are involved in various transport processes, either active or passive (reproduced from Maddrell and O'Donnell, 1992).

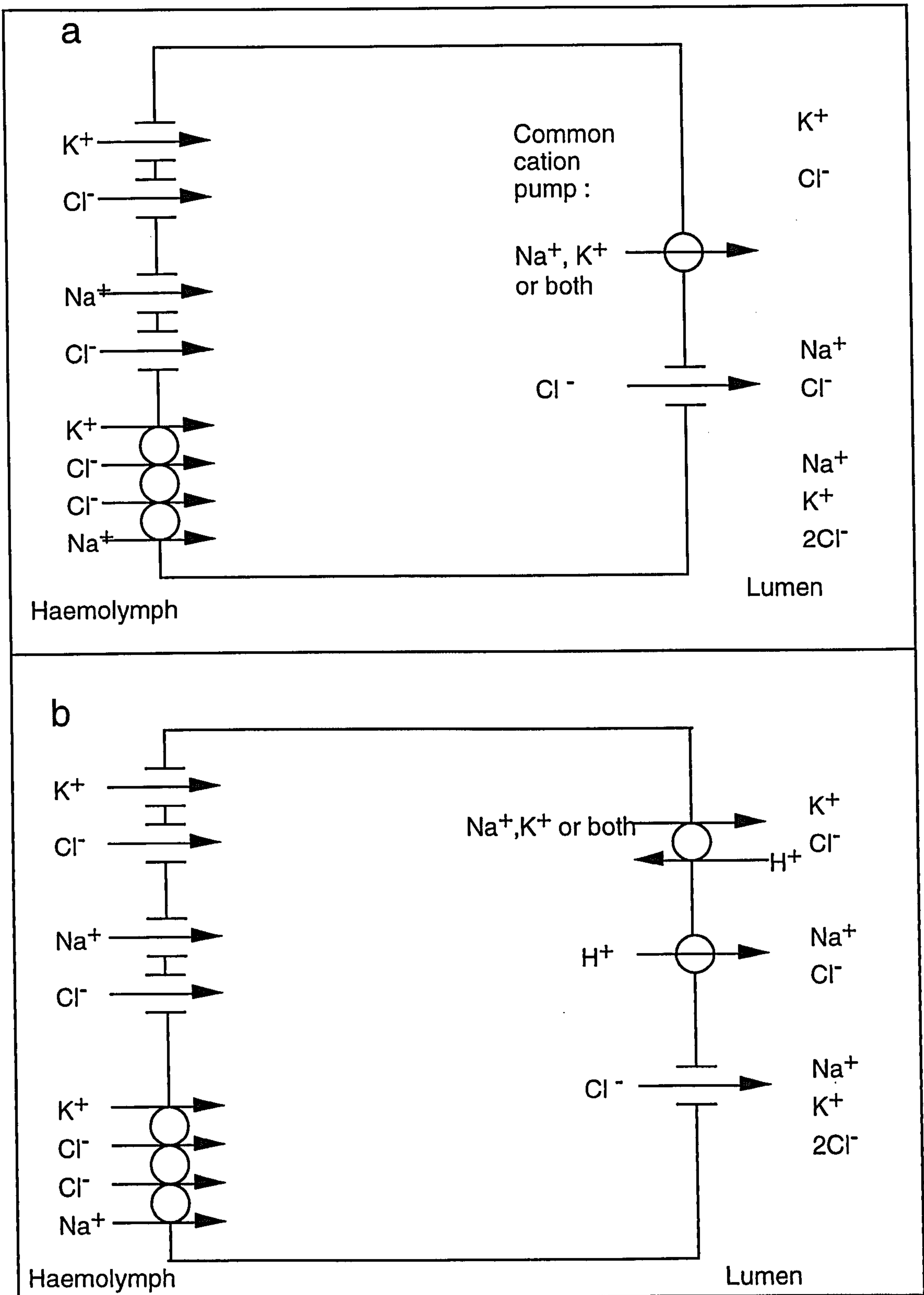


Figure 1.5. Ion transport mechanisms of Malpighian tubules.

(a) Until recently a cation pump was thought to energise the system. (b) For the present, it has been understood that the system is energised by a proton pumping apical plasma membrane V-ATPase (from Maddrell and O'Donnell, 1992).

1.5. Enhancer traps

1.5.1. Enhancers and Control of Gene expression

1.5.1.1. Gene expression

According to the central dogma of molecular biology, genes are perpetuated as nucleic acid sequences, but function by being expressed in the form of proteins through three types of processes known as “replication, transcription and translation” (Lewin, 1995). In a multicellular organism, each cell contains an identical genome but expresses a different subset of its genes depending on its growth state or environment which is also the cause of many different cell types (Watson *et al.*, 1992).

Since many processes are common to all cells, there are therefore many proteins in common, and the genes encoding them are expressed in nearly every cell. These genes are classified as "house-keeping" genes. However, there are also specific genes, expressed only in the specific types of cells, depending on some very particular mechanisms (Alberts *et al.*, 1994). One crucial factor determining the expression of the specific genes is the presence of gene regulatory proteins which recognise and bind to short stretches of double-helical DNA of defined sequence switching the specific genes on and off (Dyana and Tjian, 1985).

Unlike prokaryotic RNA polymerases, eukaryotic polymerases require the prior assembly of general transcription factors in a particular order at the promoter. These proteins are thought to work either by facilitating (positive control) or hindering (negative control) the assembly process (Watson *et al.*, 1992).

Gene expression can be mainly controlled at six steps; transcriptional control, RNA processing control, RNA transport control, translation control, mRNA degradation control and protein activity control, though there are also some other factors such as DNA methylation (Alberts *et al.*, 1995). Of these steps, the most important control point of gene expression is the initiation of RNA transcription (Darnell, 1982).

Gene expression is regulated by two general classes of cellular enhancers. One class responds to changes in the environment (inducible enhancers) For instance the enhancer of the metallothionein genes, which encode

proteins that detoxify heavy metals, is switched on by the presence of zinc or cadmium. Heat-shock genes are rapidly switched on by a shift to high temperature (Pelham, 1985). Many genes are rapidly activated in response to growth factors and hormones, indicating the regulation of inducible and tissue specific gene expression. The other class of enhancer is active only at specific times during development, or only in specific tissues, and these are known as temporal and tissue-specific enhancers (Maniatis *et al.*, 1987).

1.5.1.2. Control Elements

Two types of non-translated DNA control elements are also required for the regulation of genes that encode messenger RNA in higher eukaryotes: nearby promoters and distant enhancers. Promoters are cis-acting control elements typically located 25-30 base pairs (bp) upstream from the start site of transcription and are about hundred base pairs in length. A typical promoter includes an AT-rich region designated the TATA box and one or more sequence elements of 8 to 12 base pairs designated upstream promoter elements (UPEs) (Maniatis *et al.*, 1987). The promoters required for accurate and efficient initiation of transcription are thought to act as a site for the binding of RNA polymerase. They can be controlled both negatively by repressors or positively by effectors.

Enhancers, unlike promoters, merely amplify and are not sufficient themselves to initiate transcription. Enhancers were described as activators of gene transcription from long-range in higher eukaryotes and the first control elements conferring tissue specificity (Serfling *et al.*, 1985). Due to these properties they were considered to be different from upstream promoter elements of eukaryotic genes. The distinctive characteristic of enhancers is that they can act on *cis*-linked promoters at great distances in an orientation-independent manner and can also function downstream from the transcription unit (Gillies *et al.*, 1983; Banerji *et al.*, 1983).

Enhancers and upstream regulatory elements are composed of a modular arrangement of short sequence motifs each of which has a specific function in conferring inducibility, tissue specificity, or a general enhancement of transcription (Herr and Clarke, 1986; Maniatis *et al.*, 1987). These motifs obviously are binding sites for nuclear proteins whereby the activity of promoters and enhancers is controlled by binding

of various transcription factors to these multiple modular sequences according to one likely mechanism of action, though other models have been proposed (Atchison, 1988). However, many properties of basic components of promoters and enhancers are shared, and the mechanisms by which these components facilitate transcription may be indistinguishable. In practice, promoter and enhancer elements overlap both physically and functionally (Serfling *et al.*, 1985).

1.5.1.3. Transcription Factors

In eukaryotes, in addition to RNA polymerase II, several proteins are required for initiation and regulation of mRNA transcription. These proteins that interact with the multiple sequence elements to form the eukaryotic promoter are called transcription factors (Struhl, 1987). There are two groups of transcription factors. Some of these are general factors required for initiation at all promoters (Parker and Topol, 1984a), while others are gene specific and essential only for certain promoters (Parker and Topol, 1984b). For example, one of the general factors is a protein called TFIID, which binds to the TATA sequence in promoters. Other general factors are involved in the assembly of a multicomponent protein complex at the promoters (Watson *et al.*, 1992).

Gene-specific transcription factors are DNA-binding proteins which target principally to the upstream promoter elements (Dyran and Tjian, 1985). They are found to contain two functional domains, one for DNA-binding and one for transcriptional activation. The DNA-binding domains of transcription factors fall into several structural families based on their primary amino acid sequence such as Helix-turn-helix (homeodomain), zinc finger, leucine zipper and helix-loop-helix family. These basic structural motifs endow these proteins with the capacity to bind DNA in a sequence-specific manner outlined by Johnson and McKnight (1989).

On the other hand, the activation domains are less characterised. Cells often contain many different factors capable of binding to a single DNA sequence such as in the case of a sequence element responsible for the activation of gene transcription by the second messenger cyclic AMP. The other characteristics of transcription factors is to be modular, that is, consisted of independently functioning modules. The activation domain of one factor can be joined to the DNA-binding domain of another, and the resulting hybrid protein is fully active in cells, allowing the study of

mammalian counterparts as shown in GAL4 for example (Watson *et al.*, 1992).

In bacteria, transcription factors are thought to work either by helping RNA polymerase to bind to the promoter, or accelerating the rate at which bound RNA polymerase initiates transcription. However in eukaryotes, transcription factors might act to free DNA from nucleosomes and control elements can thereby get access to the large transcription complex (Watson *et al.*, 1992). There are several possible models for the mechanism of transcriptional activation by the transcription factors such as binding of upstream factors next to the transcriptional start site to induce a conformational change in either DNA or in other proteins in the initiation complex (Dyran and Tjian, 1985). Another likely model is forming loops in the DNA to allow distant factors to contact the transcription complex directly at the promoter (Watson *et al.*, 1992). The distribution and activity of transcription factors is dependent on both cell type and the cell cycle stage. Extracellular and environmental signals also act through second messenger pathways.

1.5.2. Importance of gene transcriptional regulation

Clearly, analysis of gene expression patterns in a multicellular organism can provide valuable insights into the development and functional organisation of the organism. Although this is in principle achievable at the RNA and protein levels (with *in situ* hybridisation or immunocytochemistry, respectively), these techniques can only be applied to already-identified genes and their products. For a more comprehensive analysis, it would be most useful if reporter genes could be introduced semi-automatically into the widest range of genes in the organism.

1.5.3. Transposable elements and enhancer traps

In the complex organisms, suitable vectors for molecular cloning occur in nature in the form of transposons that are units of DNA which can move their chromosomal positions within the chromosome or to another chromosome. Transposons have been identified in most organisms to date (Finnegan, 1992). Many transposons have been modified to produce powerful vectors with a wide range of properties and markers in a number of higher eukaryotes.

In *Drosophila*, the P family of transposable elements is the best-studied of eukaryotic non-retroviral transposon families and as a result has proved a powerful tool for analysis (Engels, 1992). These mobile elements revolutionized *Drosophila* molecular genetics, and have become important tools for gene transfer, insertional mutagenesis, enhancer trapping, and gene cloning using transposon tagging, providing a unique system with which to study gene expression (Rio, 1991). The natural complete P-element is 2.9 kb in length with two perfect 31 bp inverted repeats at either end thought to be required for transposition and four open reading frames encoding an 87kD protein known as a transposase. They have the ability to transpose at very high rates under certain genetic conditions (Engels, 1983 and 1989; Sentry and Kaiser, 1992).

Under normal circumstances P-element transposition appears to occur at very high frequency only in the progeny of a cross between a male of a P strain (containing P element) and a female of an M strain (lacking P elements) during meiosis, mobilizing the element to cause the dysgenic traits of reduced fertility, mutation induction, chromosomal rearrangement, but not in the reciprocal cross, a P x P cross or an M x M cross and only in the germline tissue (Engels, 1983). This is thought to be caused by differential splicing whereby the mRNA is only correctly spliced in the germline (Laski *et al.*, 1986).

The transposase is critical for the mobility of the P-element. The gene encoding the transposase enzyme can be replaced with DNA for other genes of choice, and also transposase enzyme provided by other means. That is, it is now known that the transposase gene does not have to be on the actual P-element itself but can also work in *trans*, on other chromosomes. This has enabled recombinant P-elements to be constructed which lack the transposase enzyme themselves. A common source of transposase is the defective P-element $\Delta 2-3$. In this P element, the $\Delta 2-3$ introns are removed which enhances transposase production as this splice is the most tightly regulated part of transposase gene expression. An internal deletion in the P-element has removed one of the 31 bp repeats essential for transposition, and permits somatic expression while leaving the transposase gene intact (Robertson *et al.*, 1988).

The technique has however been further refined to produce the modified P-element technology which enabled us to witness the development of

enhancer-trap elements. An enhancer detector is a mobilisable DNA construct containing a reporter gene that on its own is very weakly expressed; when it integrates into the genome in a region under the influence of nearby genomic transcriptional regulatory elements, gene expression can be detected by means of the reporter gene revealing the expression pattern of genes in their vicinity (O'Kane and Gehring, 1987).

1.5.4. The emergence and historical development of enhancer trap technique

The enhancer trap is a powerful new method which allows the analysis of the development of specific cell types and the identification of genes expressed more or less specifically in the cell types and tissues in *Drosophila*. Moreover the technique can be applied to many different research areas.

Cloned P-element transposition was achieved efficiently and selectively from extrachromosomal DNA to the DNA of germline chromosomes in *Drosophila* embryos, demonstrating the emergence of the first transgenic fruitfly, and provided the basis of efficient DNA-mediated gene transfer in *Drosophila* under certain genetic criteria (Rubin and Spradling, 1982; Spradling and Rubin, 1982). This was followed by "first generation enhancer trap elements" which constitute modified forms of the P-element transposon, a mobile DNA element that can insert pseudo-randomly within the *Drosophila* genome, using *lacZ* as the reporter gene. Such lines have over the years, provided a considerable variety of expression patterns (O'Kane, and Gehring, 1987; Bier *et al.*, 1989; Bellen *et al.*, 1989; Wilson *et al.*, 1989). After one year of the emergence of first generation enhancer trapping, a yeast transcription factor, GAL4, was shown to activate gene expression in the fruitfly *Drosophila* (Fischer *et al.*, 1988). The realisation of the potential of the binary system was observed in the following years providing that GAL4 was expressed from a tissue-specific promoter (Greig and Akam, 1993) and cell-type specific GAL4 expression patterns without the need for cloned promoter elements (Brand and Perrimon, 1993; Kaiser, 1993; Yang *et al.*, 1995).

1.5.5. First generation enhancer traps

First generation enhancer-traps made use of the β -galactosidase (*lacZ*) gene of *Escherichia coli* as a reporter gene. This construct also includes

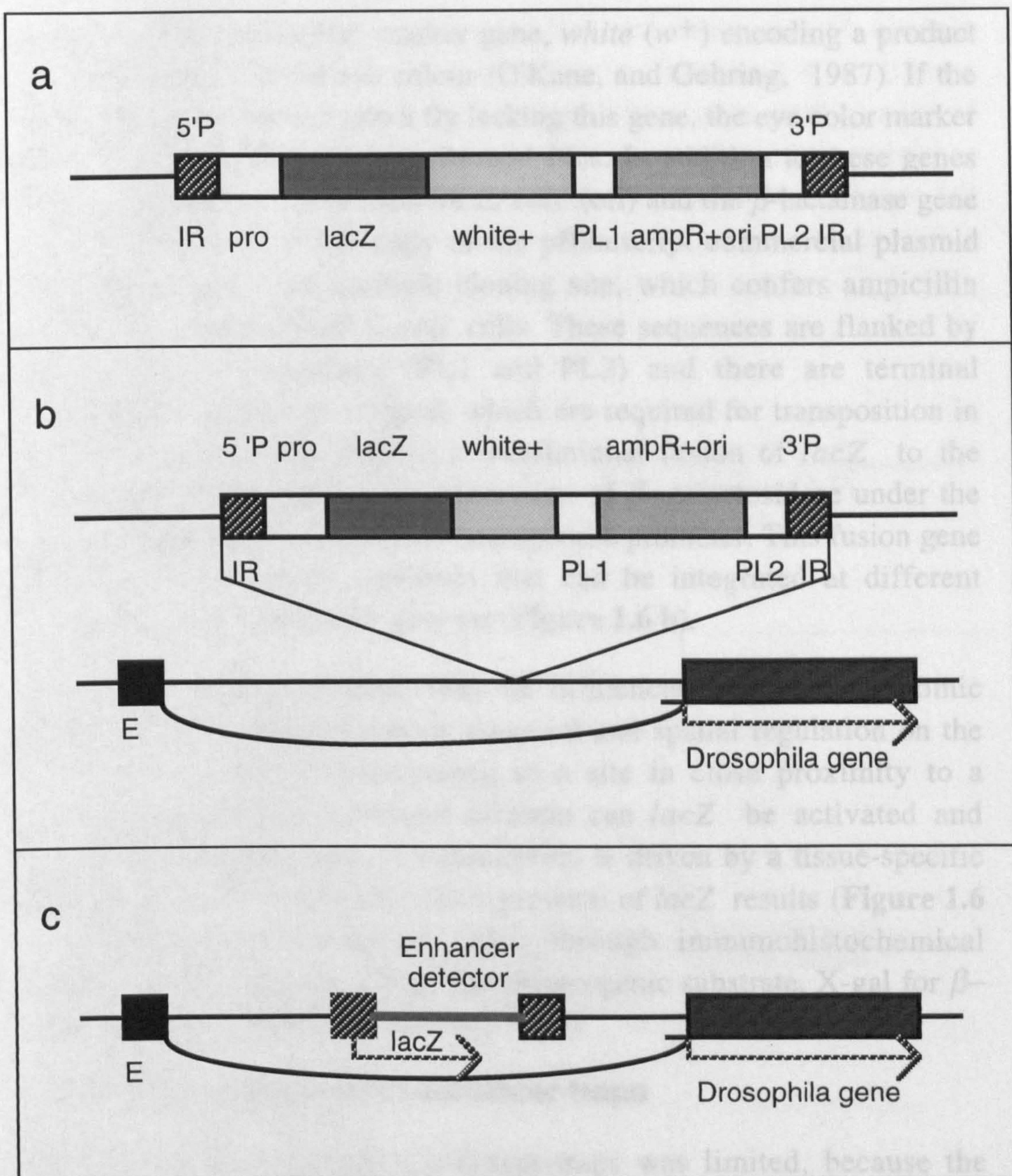


Figure 1.6. Schematic presentation of first generation enhancer traps

a) General features of first generation enhancer detector transposons in *Drosophila*. b) Influence of gene regulatory elements on the expression of β -galactosidase in a first generation enhancer detector. In this hypothetical case the enhancer normally acts on an adjacent *Drosophila* gene (a). c) When enhancer detector is located as in this case near the transcription start site of a gene, it is also transcribed and its expression can be visualised by applying the chromogenic substrate, X-gal. Abbreviations: IR, inverted repeat; PL, polylinker. (adapted with a slight modification, from Bellen *et al.*, 1990).

(Figure 1.6 a) the visible marker gene, *white* (w^+) encoding a product required for normal red eye colour (O'Kane, and Gehring, 1987). If the transposon is introduced into a fly lacking this gene, the eye color marker allows selection of stably transformed flies. In addition to these genes there is an origin of replication for *E. coli* (*ori*) and the β -lactamase gene (*amp^R*), effectively a full copy of the pBluescript commercial plasmid vector linearised at its multiple cloning site, which confers ampicillin resistance to transformed *E. coli* cells. These sequences are flanked by two polylinker sequences (PL1 and PL2) and there are terminal P-transposon sequences (striped) which are required for transposition in the construct as well. That is, a translational fusion of *lacZ* to the P-transposase gene places the expression of β -galactosidase under the control of the weak constitutive P transposase promoter. This fusion gene is part of a P-element construct that can be integrated at different locations in the *Drosophila* genome (Figure 1.6 b).

The P-transposase promoter may be influenced by nearby genomic regulatory elements that impose temporal and spatial regulation on the fusion gene. Only by transposing to a site in close proximity to a *Drosophila* promoter/enhancer element can *lacZ* be activated and β -galactosidase produced. If transcription is driven by a tissue-specific enhancer element, tissue-specific expression of *lacZ* results (Figure 1.6 c) which can be visualised either through immunohistochemical techniques or simply by using the chromogenic substrate, X-gal for β -galactosidase (O'Kane and Gehring, 1987).

1.5.6. Second generation enhancer traps

The use of first generation enhancer-traps was limited, because the reporter gene carried a nuclear localisation signal which obscures the full details of cellular geometry, a particularly severe problem in the study of neurones. On the other hand, second generation enhancer-traps have overcome some of these problems with the ability to use either nuclear or cytoplasmically-targeted reporter genes.

Second generation enhancer-traps have no histochemical reporter gene *per se*. The genes on the P-element construct include GAL4, a yeast regulatory protein which binds to a specific sequence, UASG (galactose upstream activating sequence) and activates transcription of linked genes. This has been constructed (Brand and Perrimon, 1993) in a similar

fashion to that of the *lacZ* gene in the first generation *P-lacZ* element (O'Kane and Gehring, 1987) as can be seen in **Figure 1.7 a**, i.e. it has a weak promoter, a functional copy of the *white* gene and a copy of pBluescript within the inverted repeats, containing an antibiotic resistance gene for ampicillin (*Amp^r*) and *E. coli* origin of replication (**Figure 1.7 b**). This allows the P-element to be cut out of the genome along with a piece of flanking genomic DNA using restriction enzymes. This piece

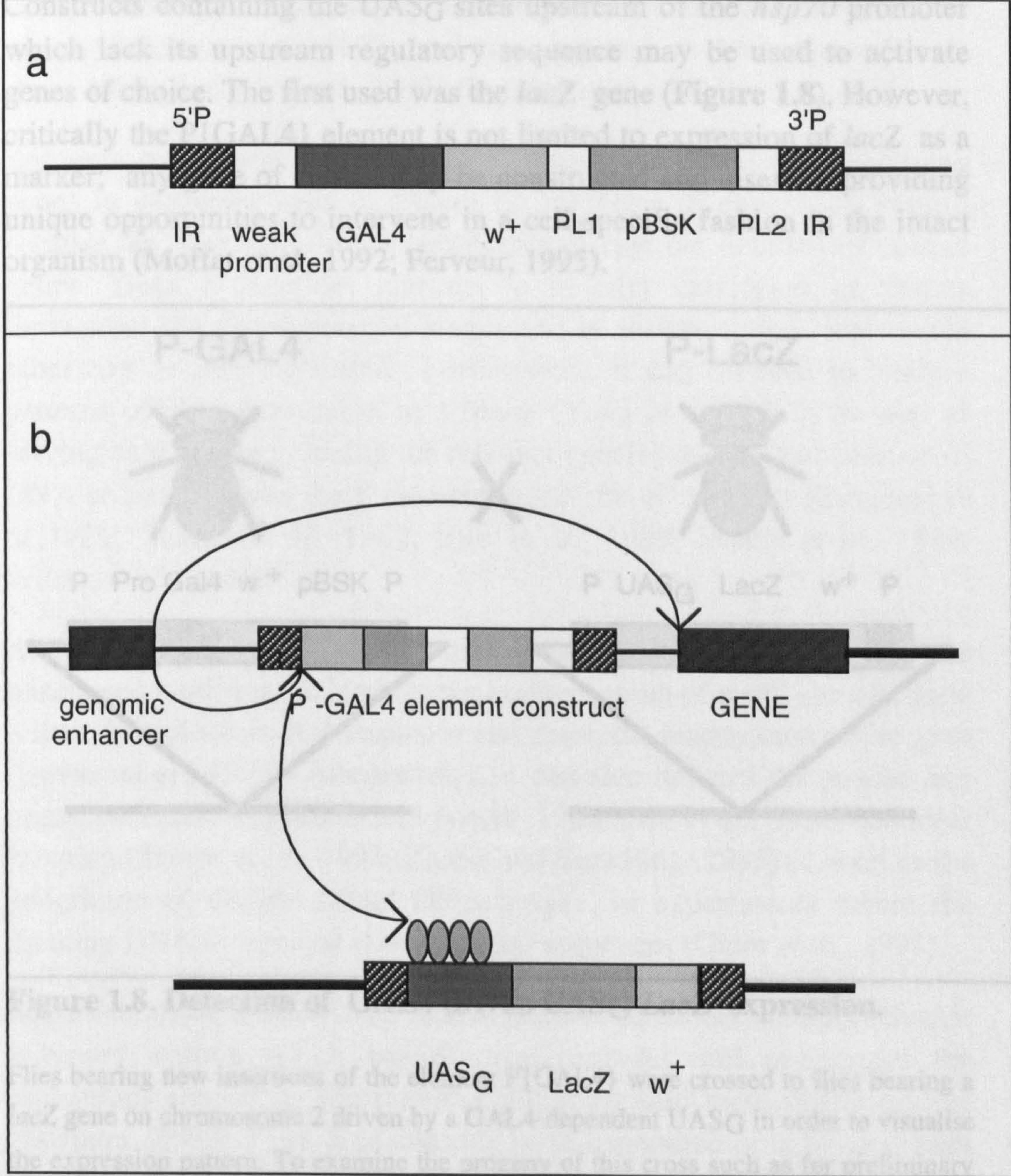


Figure 1.7. Diagram of second generation enhancer traps
 Second generation enhancer trap element (a) and its working mechanism (b).
 (reproduced with a slight modification, from Kaiser, 1993).

may then be circularised and transformed into *E. coli* in a technique known as plasmid rescue. In total this element is around 10 kb long but is still able to transpose by means of the genomic transposase source of $\Delta 2-3$ (Robertson *et al.*, 1988).

The UAS_G sites for binding GAL4 are not present in *Drosophila* so that GAL4 has no effect on normal gene transcription as far as it is known. Constructs containing the UAS_G sites upstream of the *hsp70* promoter which lack its upstream regulatory sequence may be used to activate genes of choice. The first used was the *lacZ* gene (**Figure 1.8**). However, critically the P{GAL4} element is not limited to expression of *lacZ* as a marker; any gene of choice may be constructed and inserted, providing unique opportunities to intervene in a cell-specific fashion in the intact organism (Moffat *et al.*, 1992; Ferveur, 1995).

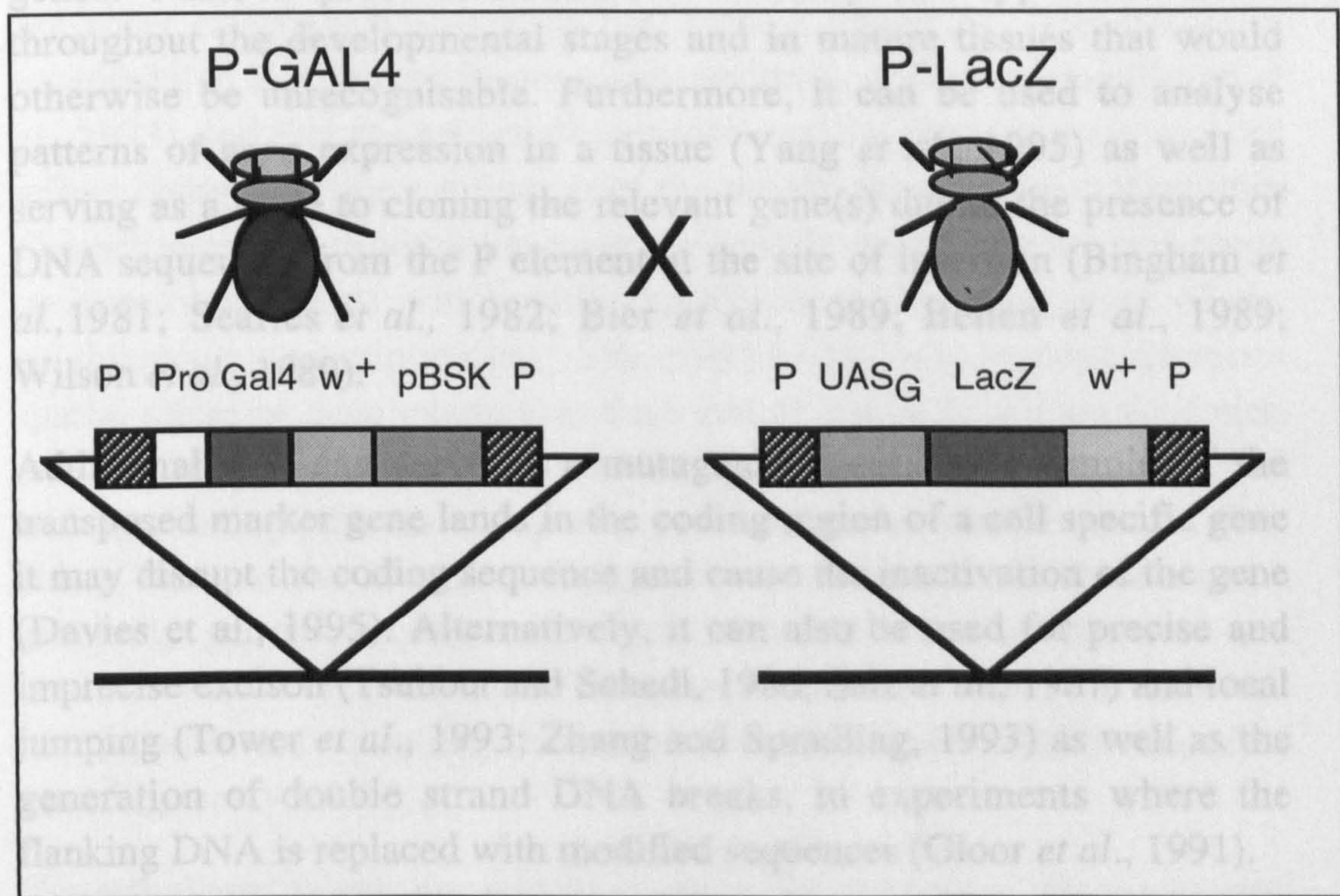


Figure 1.8. Detection of GAL4 driven UAS_G LacZ expression.

Flies bearing new insertions of the element P{GAL4} were crossed to flies bearing a *lacZ* gene on chromosome 2 driven by a GAL4-dependent UAS_G in order to visualise the expression pattern. To examine the progeny of this cross such as for preliminary screening ; after dissection of whole gut and Malpighian tubules, the larvae or adult flies are first fixed and then stained by colour solution containing the chromogenic substrate, X-gal.

1.5.7. The advantages of enhancer trapping

This “enhancer trapping” technology has provided valuable information on spatial patterning of gene expression (Bertram *et al.*, 1992; Hartenstein and Jan, 1992; Riesgo-Escovar *et al.*, 1992). Chromosome walks from marked P-elements have led to several important genes being characterised (Doe *et al.*, 1991; Nose *et al.*, 1992; Schubiger *et al.*, 1994). Recently, this technique has been extended to vertebrates, such as mouse (Soininen *et al.*, 1992), and to plants, such as *Arabidopsis* (Devic *et al.*, 1992; Lindsey *et al.*, 1993; Topping *et al.*, 1994).

Enhancer trap insertions provide an important method for identifying genes in particular cells or tissues on the basis of expression pattern because the insertions do not necessarily disrupt the function of normal genes. Thus, it provides markers to identify cell types or tissues throughout the developmental stages and in mature tissues that would otherwise be unrecognisable. Furthermore, it can be used to analyse patterns of gene expression in a tissue (Yang *et al.*, 1995) as well as serving as a route to cloning the relevant gene(s) due to the presence of DNA sequences from the P element at the site of insertion (Bingham *et al.*, 1981; Searles *et al.*, 1982; Bier *et al.*, 1989; Bellen *et al.*, 1989; Wilson *et al.*, 1989).

Additionally, it can serve as a mutagenic agent; for example, if the transposed marker gene lands in the coding region of a cell specific gene it may disrupt the coding sequence and cause the inactivation of the gene (Davies *et al.*, 1995). Alternatively, it can also be used for precise and imprecise excision (Tsubota and Schedl, 1986; Salz *et al.*, 1987) and local jumping (Tower *et al.*, 1993; Zhang and Spradling, 1993) as well as the generation of double strand DNA breaks, in experiments where the flanking DNA is replaced with modified sequences (Gloor *et al.*, 1991).

In particular, the GAL4/UAS second generation enhancer trap method is a binary system which has extra advantages and overcomes the difficulties caused by the previous enhancer trap method (Brand and Perrimon, 1993). First, it enables the rapid generation of individual lines in which ectopic expression of a transgene can be directed to many different tissues and cell types. Second, it separates the transgene from its transcriptional activator in two distinct transgenic lines, providing a useful tool for insertion of potentially damaging constructs without the

development of "modifiers", that prevent propagation, over extended periods. Third, it activates the target gene in different cell-and tissue-types only by crossing a single line carrying the desired target to a library of activator expressing strains.

However, enhancer detection simultaneously allows the identification of many cell or tissue-specific regulatory elements. If elements that regulate expression in cells of particular interest are determined, they may be subsequently used to drive expression of cellular modifiers permitting either ablation or alteration of the fate of particular cells allowing specific questions to be asked concerning the function of these cells in the organism (Moffat *et al.*, 1992, Ferveur *et al.*, 1995; O'Dell *et al.*, 1995).

1.5.8. The difficulties with enhancer trap

Enhancer trapping, however, is not without problems. It is generally accepted that P-elements do not insert randomly in the genome (Smith *et al.*, 1993), and that the pattern of expression of the reporter gene may not reflect completely the expression of the neighbouring transcription unit. The enhancer trap reporter gene may be heavily influenced at short-range by control elements which only contribute to the expression of the neighbouring transcription unit. The effect of this is to report a narrower spatial range of gene expression than that of the authentic transcription unit. For this reason, attempts to identify transcription units associated with interesting patterns of reporter gene expression can be complicated, either because the neighbouring gene was subsequently shown to be relatively generally expressed, or because the enhancer detector had inserted into the genome at so great a distance that the relevant transcription unit either could not be found, or could not be identified unambiguously.

Nevertheless, when an enhancer detector reports a clear-cut and repeatable pattern of expression, this implies that it is under the influence of a control element or combination of control elements which are capable of delineating a domain of gene expression within the organism. This is made even likelier if more than one independent insertion event reports the same pattern of expression. In this way, the screening of a large number of independent enhancer-trap lines provides a useful indication of the functional boundaries within a tissue under study. This technique is quite independent of the precise identity of the neighbouring

transcription units, although it may subsequently lead to their characterisation.

1.6. Aims of the study

The aims of the study was to investigate and analyse domains of gene expression of the tubules with enhancer-trap technology, and to reconcile these data with the developmental, morphological and physiological data which have recently become available to provide both a validation of the use of enhancer traps in the identification of functionally significant boundaries, and to provide new avenues for further study of the tubules at the morphological, physiological and molecular levels.

Due to the fact that enhancer trapping is a new technique used to identify genes expressed more or less specifically in the cell types and tissues on the basis of expression pattern, the aim in terms of molecular biology of ion transport was also to find and analyse some interesting and specific ones expressed in the Malpighian tubules, that is, some *LacZ* reporter gene expressions restricted to certain parts or cell types of tubules that may play a role in primary urine formation, ion transport or signal transduction pathway which will allow further manipulation and characterization at a molecular level; such as the cloning, mapping and studying the function of related genes in both *Drosophila* and vertebrates.

Chapter 2: Materials and Methods

2.1. *Drosophila* methods

2.1.1. General principles in generating P{GAL4} lines

General principles adopted in generating lines were essentially as described elsewhere (Bellen *et al.*, 1989; O’Kane and Gehring, 1987; Smith and O’Kane, 1991; Spradling and Rubin, 1982). A total of 1500 P{GAL4} lines were generated in our lab in essentially the Canton-S genetic background as described by Brand and Perrimon (Brand, and Perrimon, 1993). GAL4 is a yeast transcription factor that when expressed in *Drosophila* does not appear to recognise or activate any *Drosophila* promoter, that is, there is no known endogenous target for GAL4 in the *Drosophila* genome. Its pattern and timing of expression depend upon the genomic context of the inserted P{GAL4} element. GAL4 can be used to drive expression of any genes linked to the GAL4-responsive promoter, UASG, either with the secondary reporter for GAL4 activity as a homozygous(although the resulting generation could be heterozygous) second chromosome insertion in the original line UASG-*lacZ* , or any other genes of choice. There is no detectable *lacZ* or any other secondary reporter expression in the absence of GAL4.

2.1.2. The mutagenesis and particular crossing scheme

As mentioned earlier the transposase source chosen for the mutagenesis is $\Delta 2-3$ on chromosome 3 (Robertson *et al.*, 1988). This modified P-element was shown to be remarkably stable, as the 31bp inverted repeats (IR) which are required for transposition are defective. The $\Delta 2-3$ introns are removed which enhances transposase production as this splice is the most tightly regulated part of transposase gene expression. The stability of the transposon allows it to be treated not as a transposable element but as a genetic factor which can be removed by genetic crossing. The $\Delta 2-3$ strain most commonly used in Glasgow is balanced by the TM6B balancer chromosome and is linked to the *Drop* (*Dr*) dominant visible marker phenotype. The balancer allows potentially lethal mutations to be propagated as heterozygotes without selection in each generation, and the *Dr* marker provides easy selection for crossing out the transposase source. The P{GAL4} element was maintained *en*

masse in a white (w^-) background over a *Curly of Oster* balancer chromosome (*CyO*).

To perform the cross, P{GAL4}, *CyO* virgin females are mated to $\Delta 2-3$ males *en masse*.The resultant F1 progeny could be selected by their *Dr* phenotype (from the $\Delta 2-3$ line used), wild type eye colour (from PGAL4) and curly wings (from the *CyO*) balancer chromosome. Males of these are crossed to virgin white females (w^{1118} is used). The F2 progeny are selected for loss of the *Dr* phenotype, loss of the *CyO* chromosome and wild type eye colour. The F2 cross is carried out in vials containing one or two males and between five and eight virgin white females.

The particular crossing scheme for mutagenesis is shown in **Figure 2.1**:

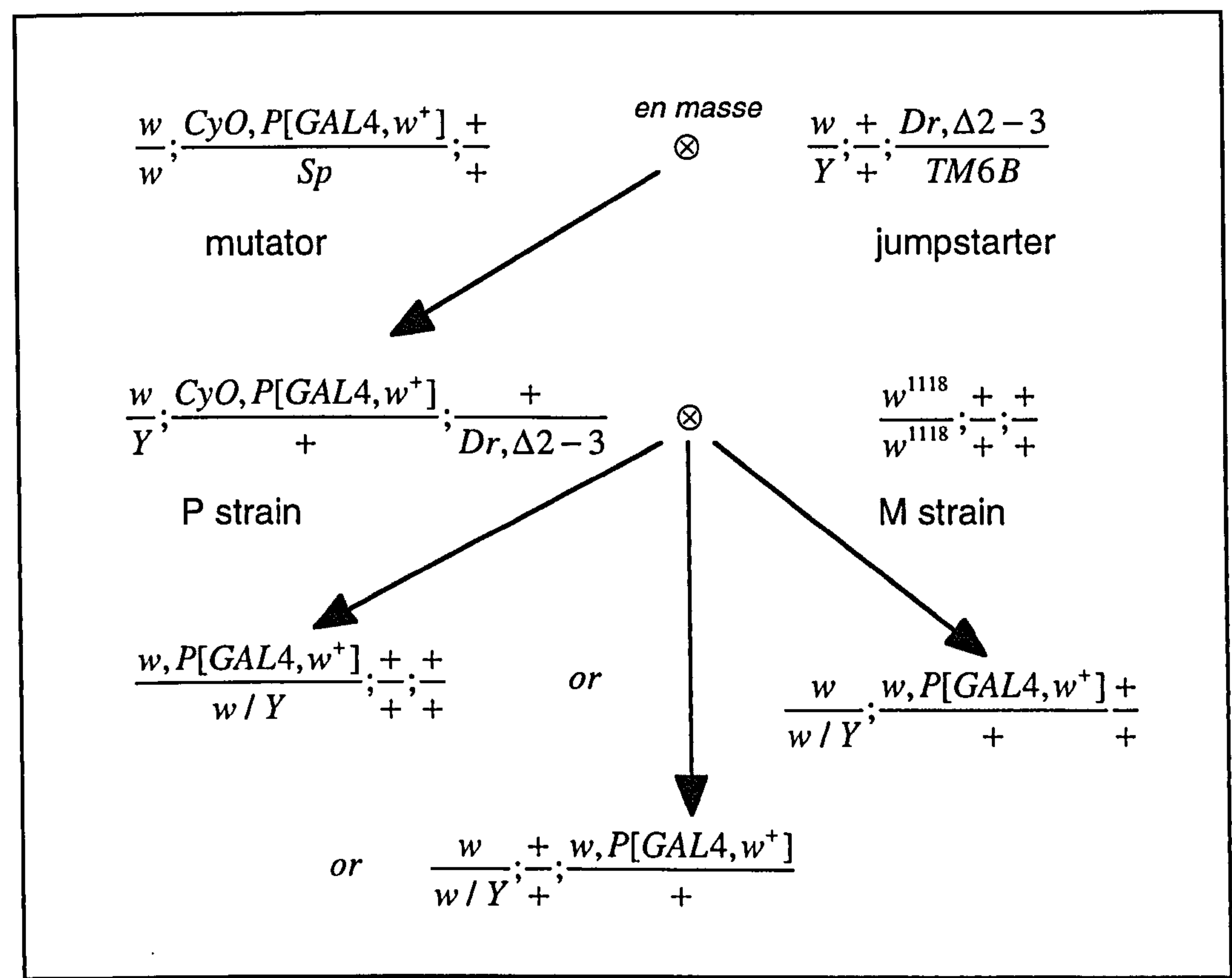


Figure 2.1. Crossing scheme for the generation of enhancer-trap lines.

A line carrying the P{GAL4} element was mobilised by crossing virgins to males carrying the *Dr*, $\Delta 2-3$ source of transposase in a w^- background. Jump-start male progeny with both *CyO* and *Dr* markers were crossed with w^- females, and their progeny segregated and selected for loss of *CyO* and *Dr*. In principle, insertions on all chromosomes can be recovered.

2.1.3. Fly stocks and rearing conditions

The lines used for this P{GAL4} mutagenesis were kind gifts of Andrea Brand and Tim Tully. The resulting P{GAL4} lines were maintained in vials by selecting against white eye colour, and most lines eventually became homozygous. The secondary reporter for GAL4 activity was a homozygous UASG-*lacZ* insertion on the second chromosome, donated by Andrea Brand (Brand and Perrimon, 1993). There is no detectable *lacZ* expression in the absence of GAL4. The genetic background of all lines generated was Canton-S. Lines outbred with Canton-S wild-type have also been examined and show no obvious differences from the inbred stocks with respect to their expression patterns. P{GAL4} lines were initially screened for expression in the larvae and adult Malpighian tubules from the F1 progeny of P{GAL4} X UASG-*lacZ*, by staining with X-gal, a chromogenic substrate for β -galactosidase, which converts it to a blue product.

Drosophila were maintained in standard conditions (Ashburner, 1989), on a 12h dark : 12h light cycle on standard cornmeal/yeast/agar medium at 23°C-25°C and 35%-45% relative humidity. Fly density was not strictly controlled but flies were not over-crowded and were shaken over at regular intervals. Fly crosses were of two males and three females per vial. When large numbers of flies were required crosses were carried out in bottles with approximate numbers of males and females. Before the next generation hatched, parent flies were removed. Unless otherwise described, histology was performed on adult flies of age 2-14 days and third instar larvae.

2.2. Visualisation techniques for detection of expression of GAL4 Driven UASG-*lacZ*.

The expression of GAL4 is not itself easily detectable, but on crossing to lines carrying *LacZ* downstream of the UASG recognition sequence of the GAL4 protein, the β -galactosidase reporter gene is strongly expressed in those cells where GAL4 has been expressed. To accomplish this, virgin females of line (UASG-*lacZ*) were crossed to males of each enhancer trap line; and embryos, larvae or adults collected and β -galactosidase detected as described below. There are limitations inherent in this cross; only half of the progeny could be expected to contain both P{GAL4} and P{UASG-*lacZ*, w^+ } and thus show directed β -

galactosidase activity. For this reason, at least 6 embryos or larvae were studied for each line, implying that only 1 in 2^6 lines would be discarded wrongly. For adults, the *white*⁺ marker of P{UASG-*lacZ*, *w*⁺} was used; only red-eyed flies were analysed, as all should contain one copy of both P-elements. There is an additional problem; where the P-element is inserted on the X or Y chromosomes, the cross would erroneously imply a sex-specific expression pattern. However, this problem was infrequently encountered in this study.

2.2.1. Dissection techniques

Larvae or adults were immersed in PBS in a Sylgard (Dow Corning Ltd) lined petri dish and dissected by gripping neighbouring abdominal tergites laterally with two pairs of fine watchmakers' forceps and tearing the body wall open. For adult flies, the wings and legs were removed prior to immersion in solution in order to prevent them from floating. The two halves of the body were then drawn apart, uncoiling the alimentary canal, and flies were pinned into the dish through the head and tail, and staining performed for *X-gal*. For immunocytochemistry however, further dissection was necessary to free the tubules from their attachments, and then each pair of tubules (anterior and posterior) was cut free at the junction with the common ureters which connect them to the alimentary canal.

2.2.2. Fixation Methods

For fixation both glutaraldehyde and paraformaldehyde were used successfully. For preliminary screening performed with the chromogenic substrate, *X-gal*, fixation was achieved using 1% glutaraldehyde in the phosphate saline buffer (PBS: 1.3 M NaCl, 0.07 M Na₂HPO₄·7H₂O, 0.03 M NaH₂PO₄·H₂O, Ashburner, 1989). For immunocytochemical detection of β -galactosidase, 4% paraformaldehyde in PBS (pH 7.3) was used as a fixative, because glutaraldehyde increases background autofluorescence. Paraformaldehyde was equally suitable both for detection of *X-gal* and for immunocytochemistry in embryos.

2.2.3. Detection of β -galactosidase using X-gal

Preliminary screening was performed by colorimetric detection of β -galactosidase according to established protocols (Ashburner, 1989).

This had the advantage of speed and sensitivity, but the spread of the slightly soluble blue reaction product was capable of obscuring the finer details of expression. Alimentary canals and tubules were dissected and pinned into a Sylgard lined petri dish as described above. Then they were fixed for 10-15 minutes in 1% glutaraldehyde in PBS, rinsed in PBS, then transferred to staining solution (Ashburner, 1989) and stained for 30-120 minutes in the dark at 37°C in a moist box. The staining solution was 0.2% Xgal (diluted from an 8% stock solution in DMSO) in pre-warmed FeNaP buffer (FeNaP is 10mM NaH₂PO₄.H₂O, 10mM Na₂HPO₄.2H₂O, 150mM NaCl, 1mM MgCl₂.6H₂O, 3.1mM K₄(Fe²⁺+CN)₆, 3.1 mM K₃(Fe³⁺+CN)₆, 0.3% Triton X-100, pH 7.4). The staining solution was made freshly, aliquoted and kept dark. The reaction was stopped by rinsing with PBS, and alimentary canals and tubules were viewed at low power under incident light with a Wild M8 stereo microscope, or at higher power by transmitted light in a Leitz Ortholux microscope.

2.2.4. Immunocytochemistry

2.2.4.1. Poly-L-lysine treatment of slides

Microscope slides were washed with ethanol and dried with a tissue, then a square shaped area was outlined in the centre of the slide with a wax pen (Dako). Poly-L-Lysine solution of 100 µg/ml in working concentration was added sufficient to cover the area (approximately 100-200 µl) and left at the room temperature for 30-60 minutes without letting them dry out. At the end of this period the slides were washed extensively under tap water and a sufficient amount of PBS or any other solution of interest such as *Drosophila* saline or Rinaldini's saline was added (roughly 100-150 µl) to the outlined area. The tubules dissected and cut from the middle of the ureter were transferred to the drop and stuck readily on the Poly-L-Lysine coated surface of the slides.

2.2.4.2. Immunocytochemical detection

The protocol used was based on one kindly supplied by Helen Skaer. Intact tubules or alimentary canals were dissected under PBS and touched onto poly-L-lysine coated microscope slides, to which they adhered strongly, then fixed in 4% paraformaldehyde for 30 min, rinsed twice in PBS, permeabilised in PBS-TX (0.3% Triton X-100 in PBS) washing

twice, blocked at least for 1 hour in PAT (PBS containing 0.5% Sigma cold fraction V bovine serum albumin and 0.3% Triton X-100). They were incubated overnight with rabbit polyclonal anti- β -gal antibody (Cappel) diluted 1:2000 in PAT. The next day tubules were washed three times in PAT for 1 hour; blocked for two hours in PAT with 2% normal goat serum (SAPU); then incubated overnight with secondary antibody (fluorescein-labelled goat anti-rabbit IgG; Vector Labs) diluted 1:250 in PAT containing 2% normal goat serum; washed three times for 1 hour in PAT, and once for 5 minutes with PBS. All of the above procedures were carried out at room temperature except for the first antibody incubation which was at 4°C. Stained tubules were mounted in VectaShield mounting medium (Vector).

2.2.4.3. Primary Antibody

The primary antibody used in this study was the Cappel (USA) rabbit polyclonal antibody. It was used successfully at 1:2000 dilution in PAT usually; however, occasionally similar successful results were achieved at 1:1500 dilution. Each slide required about 150 μ l of working solution.

2.2.4.4. Secondary Antibody

Two kinds of good secondary antibody were used for the Cappel rabbit polyclonal antibody. A fluorescein-labelled goat anti-rabbit IgG antibody from Vector Labs at 1:250 dilution in PAT was the most commonly used one in this study. However a Texas Red-labelled sheep anti-rabbit IgG antibody from Molecular Probes at 1:500 dilution was also used very successfully.

A biotinylated antibody from Vector was also used, in which case it was visualised with the Vectastain ABC Elite kit used according to the manufacturer's instructions, which uses peroxidase conjugated to avidin that binds to the biotinylated antibody. The peroxidase is then detected by adding diaminobenzimidine (DAB), a chromogenic substrate which produces a dense black precipitate on reaction.

2.2.5. Counterstaining with ethidium bromide

To provide more precise information on the position of cell boundaries, or the relative numbers of different cell types, fluorescently labelled tubules as described above were counterstained with ethidium bromide at

twice, blocked at least for 1 hour in PAT (PBS containing 0.5% Sigma cold fraction V bovine serum albumin and 0.3% Triton X-100). They were incubated overnight with rabbit polyclonal anti- β -gal antibody (Cappel) diluted 1:2000 in PAT. The next day tubules were washed three times in PAT for 1 hour; blocked for two hours in PAT with 2% normal goat serum (SAPU); then incubated overnight with secondary antibody (fluorescein-labelled goat anti-rabbit IgG; Vector Labs) diluted 1:250 in PAT containing 2% normal goat serum; washed three times for 1 hour in PAT, and once for 5 minutes with PBS. All of the above procedures were carried out at room temperature except for the first antibody incubation which was at 4°C. Stained tubules were mounted in VectaShield mounting medium (Vector).

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2.2.5. Counterstaining with ethidium bromide

To provide more precise information on the position of cell boundaries, or the relative numbers of different cell types, fluorescently labelled tubules as described above were counterstained with ethidium bromide at

1-5 ng/ml (diluted in PBS from a 10 mg/ml stock solution in water) for 1 min before viewing under a Leitz fluorescence microscope, using fluorescein optics. In this way, it was possible to count the number of nuclei from the tip of the tubule to the edge of a fluorescently marked compartment in a number of different preparations, and so obtain numerical data. Either primary (Type I) or stellate (Type II) cells were counted, as appropriate to the subregions, and data were analysed with Microsoft Excel (version 4.0) programme and statistical data were obtained. Such data are presented below as means \pm SEM, with the number of preparations measured in brackets. Where appropriate, statistical significance was tested using Student's *t* test (two-tailed), taking $P < 0.05$ as the critical level.

2.2.6. Mounting of the preparations

For microscopical inspection by transmitted light, microscope slides carrying stained tubules were drained and either PBS or glycerol added, and a coverslip overlaid. To ensure that tubules were not crushed in semipermanent preparations, it was normal to stick fragments of coverslip (22X40 mm thickness no. 1 BDH) to the slide using a melted (55°C) glycerol-gelatin mountant (Sigma) before applying the coverslip.

For fluorescent preparations, Vectashield liquid mounting medium (Vector Labs) was used most successfully. In that case, stained tubules with fluorescent secondary antibody were mounted between sealed coverslips. A drop of mountant was placed on the centre of a glass microscope slide. A small square (22mm square, thickness 1.0 BDH) coverslip was stuck to either end of the slide using Glycerol-Gelatine (Sigma). A long coverslip (22X64 mm thickness 1.0 BDH) was then placed on top and stuck to either of the smaller coverslips again using Glycerol-Gelatine. The coverslip was then sealed along either edge using Glycerol-Gelatine.

For peroxidase/DAB stained preparations, glycerol was used as a mountant, simply by placing a sufficient amount on the centre of the slide's outlined area, then just a small drop of glycerol-gelatine was put to each corner of the restricted area, and then either a small square coverslip or slightly larger rectangular medium size coverslip was stuck to the corners and sealed using glycerol-gelatine.

2.2.7. Vital detection and isolation of a single stellate cell

Recently, lipophilic substrates for β -galactosidase have been developed which permit vital staining. Tubules were dissected in PBS, then transferred into Rinaldini's saline solution and incubated without further pretreatment in Rinaldini's saline containing 1:1000 C2FDG (Molecular Probes) for 1-2 hours (diluted from an 0.63% stock solution in 20% DMSO into distilled water). (Rinaldini's $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free saline, which reduces adhesion of cells in acute culture, is prepared as a 5x stock: NaCl, 40 g/l; KCl, 1 g/l; $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.25 g/l; NaHCO_3 , 5 g/l; glucose, 5 g/l.)

For isolation of a single stellate cell, vitally stained adult tubules from the lines of C724 and/or C710 which marks only stellate cells in the tubules were treated with "Dispase type II" (Boehringer-Mannheim) in *Drosophila* saline at 1 mg ml⁻¹ for 10-30 minutes at 32°C, and then viewed under fluorescence or confocal optics.

2.2.8. Vital detection with Green Fluorescent Protein (GFP)

P{GAL4} lines were crossed with the flies carrying UASG-Green Fluorescent Protein (GFP). The resulting progeny were analysed and viewed under epifluorescence, after dissection and adhesion to Poly-L-Lysine coated slides as described in section 2.2.4.

2.2.9. The effect of temperature on variegated expression

Some specific lines were crossed with UASG-*lacZ* lines and incubated at 18°C and 25°C respectively to hatch the coming generation (F1) and analysed for the heterogeneity of GAL4 expression pattern.

2.2.10. Organic Solute Transport Assay

Freshly-dissected adult tubules were stuck to Poly-L-Lysine coated slides in PBS. Then they were incubated in Rhodamine 123 solution in 1:500 dilution from a 1mg/ml⁻¹ stock solution for 20 s; or in 0.1 $\mu\text{l}/\text{ml}$ ethidium bromide diluted from 10 mg/ml for 30 s, or in 0.25 $\mu\text{l}/\text{ml}$ for 15 s, and replaced again with PBS and viewed under fluorescence optics.

For the longer incubation assays, tubules were incubated with Rhodamine 123 for 10-20 minutes then Rhodamine was rinsed off and tubules were left in PBS for 1-2 hours. They were viewed one and two hours later.

2.2.11. Alkaline Phosphatase Distribution

Freshly dissected adult tubules were stuck to Poly-L-Lysine coated slides and fixed in 4% paraformaldehyde in PBS for 30 minutes. Alkaline Phosphatase activity was then detected histochemically using nitroblue tetrazolium/X-phosphate (NBT/X-phosphate), reagents from the Boehringer-Mannheim DIG labelling and detection kit. Samples were incubated in a fresh-made solution of PBS containing 45 µl of NBT and 35 µl of X-phosphate in 1 ml of PBS at room temperature for 2-12 hours. Reaction was stopped by rinsing with PBS, and photographs taken.

2.2.12. Conventional Microscopy.

At the preliminary screening, alimentary canals and tubules were viewed at low power under incident light with a Wild M8 stereo microscope, or at higher power by transmitted light in a Leitz Ortholux microscope for finer resolution.

For immunocytochemical examinations, slides were viewed by epifluorescence in a Leitz Ortholux microscope either through a fluorescein filter when a fluorescein labelled goat anti-rabbit IgG antibody from Vector Labs was used; or a Rhodamine filter when a Texas-Red labelled sheep anti-rabbit IgG antibody from Molecular Probes was used as a secondary antibody, and photographed with a Wild microscope camera using Ektachrome 200 daylight (slide) film.

2.2.13. Confocal microscopy

Whole-mount stained tubules were examined with a Molecular Dynamics Multiprobe laser scanning confocal microscope. Excitation (480 nm) and emission (530±15 nm) barrier filters were used as appropriate for the labels of the secondary antibody.

For detailed work, optical sections were taken at 1 µm intervals through the depth of the tubule (around 35 µm).

2.2.14. Reconstruction and image processing

Reconstructions were performed using the programme 'ImageSpace 3.1' (Molecular Dynamics). Pseudo-colour was added to the reconstructed view using the programme 'NIH-Image' (National Institutes of Health, Washington).

2.3. Developmental analysis

Developmental expression patterns were studied in tubules either by staining with X-gal, or by immunocytochemistry, using protocols based on those described above for adults.

The detection of expression using X-gal in embryos was performed as follows: Eggs laid on apple juice (or grape juice) media with a small smear of yeast paste to encourage oviposition (Ashburner, 1989) were collected from the surface by a paint-brush into a sieve, then washed with distilled water to remove any yeast paste, and dechorionated in 50% bleach for 1-3 minutes. Embryos were then fixed at the heptane-fix (4% paraformaldehyde in PBS) interface in a universal, while shaking them occasionally. Embryos were then transferred into an eppendorf and blocked with PTB (PBS containing 0.1% Triton-X 100, 0.1% BSA) twice, and later washed with PTW (PBS with 0.3% Tween 80) three times. Finally embryos were incubated in colour solution (see section 2.2.3) at 37°C for two hours.

Immunocytochemistry of embryo whole mounts was carried out according to the protocol kindly provided by H. Skaer (pers. com.).

Embryos were similarly collected from the media by a paint-brush into a small sieve and fixed at a heptane-fix interface shaking horizontally on the shaker at room temperature for 20-30 minutes. After blocking with PBS-TX-BSA the primary antibody was added overnight in 1:10 000 dilution (Cappel). Following three 20 min washes with PBS-TX or PBS-TX-BSA, and blocking with PBS-TX containing 1:50 horse serum, embryos were incubated with biotinylated secondary antibody for 30-60 minutes. Finally, colour was developed using the ABC Elite kit and diaminobenzidine (DAB), and samples were dehydrated through a graded alcohol series and mounted in DPX medium.

2.4. Ablation of stellate cells

The stellate specific P{GAL4} lines were crossed with the flies carrying UASG-ricin and UASG-lacZ (Moffat *et al.*, 1992). The progeny were heat-shocked at 30°C for ten days or 37°C for 15 hours. Then GAL4 directed *lacZ* or GFP expression was detected either with *X-gal* or with an antibody against β -gal, or directly under epifluorescence (for the GFP

x UASG-ricin cross), or assayed physiologically with the tubule secretion assay (Dow *et al.*, 1994a).

2.5. Preliminary molecular characterisation of some of the candidate genes specifically expressed in Malpighian tubules

Around ten lines with tubule-specific expression patterns were subjected to a preliminary molecular characterisation. For this the following steps were followed.

2.5.1. Plasmid rescue

Plasmid rescue was performed according to the protocol provided from Hafen lab with a slight modification (Walter, Jenni M. and Hafen E., 1991).

2.5.1.1. Preparation of genomic DNA

Around 15-20 adult flies were collected into an eppendorf and chilled on ice for about 5 minutes, then 300µl of modified lysis buffer (100 mM Tris pH 8.5, 80 mM NaCl, 5% sucrose, 50 mM EDTA pH 8.0, 0.5% SDS, 0.15 mM spermine/spermidine mix) was added, the flies were ground for 1 minute with a plastic pestle and mortar, then incubated at 70°C for 30 minutes, KOAc was added to a final concentration of 1M, and the samples were placed on ice for 30 minutes. Following the centrifugation for 15 minutes at 4°C, the supernatant was transferred to a new centrifuge tube (1.5 ml) and precipitated with 0.6 volumes of isopropanol (about 200 µl), mixed by inversion and incubated for 5 minutes at room temperature. After centrifugation at 13000 rpm for 10 minutes, the pelleted genomic DNA was washed with 500 µl of 70% (v/v) ethanol and dried.

2.5.1.2. Digestion and ligation of genomic DNA

For digestion, the dried genomic DNA pellet was resuspended in 88-89 µl of distilled water and 10 µl of 10X React 2 buffer (Gibco BRL), then 1-2 µl of the endonuclease restriction enzyme of choice was added, and the reaction incubated overnight at 37°C in a water bath.

The digestion was stopped by heating the tubes to 70°C for 15 minutes; then ligation was performed by adding to the same tubes 89 µl of distilled

water, 10 µl of 10X modified ligase buffer (300mM Tris-HCl pH 7.4, 100mM MgCl₂, 200 mM DDT and 20 mM ATP), 1 µl of T4 DNA ligase (BRL), and incubated at room temperature overnight.

2.5.1.3. Transformation into *E. coli*

The chemical transformation method was used. For this, first competent cells of *E. coli* from either the XL1-Blue or DH5- α strains were prepared as follows:

A single colony was picked from a plate, and used to inoculate an overnight culture, which was grown for 16-20 hours with shaking at 37°C, and 1 ml of it was transferred into 100 ml of L Broth in a 250 ml flask. The culture was incubated for approximately 3 hours at 37°C with vigorous shaking to an optical density (OD₆₀₀) of 0.5 (between 0.45-0.6 is acceptable). The cultures were then cooled to 0°C by storing on ice for 10-15 minutes, and the cells recovered by centrifugation at 4000 rpm for 10 minutes at 4°C in a Sorvall GS3 rotor. The media were decanted from the cell pellet, and the last traces drained by standing the tubes (50 ml polypropylene Falcone 2070) for 1 minute in an inverted position. The cell pellet was resuspended in 10 ml of ice-cold 0.1 M CaCl₂ and later the cells were pelleted once more in the same way in 2 ml of ice-cold 0.1 M CaCl₂ for each 50 ml of original culture. At this point the cells (now competent) were used either freshly, or were frozen in 200 µl aliquots by immersion in liquid nitrogen, for later use.

Transformation was carried out by gently transferring 200 µl of competent cells to a sterile 1.5 ml microfuge tube using a chilled, sterile pipette tip and the ligation mixture or plasmid (about 100 ng in a volume of 20 µl or less) was added. The contents of the tubes were mixed by stirring very gently, stored on ice for 30 minutes, then heat shocked by transferring to a rack placed in a 42°C circulating water bath for 90 seconds, and transferring rapidly to an ice bath for 1-2 minutes. After the addition of 800 µl of 2X YT or L-Broth medium, the cultures were incubated for 45-60 minutes at 37°C in a rotary shaker, agitating the cells gently, and then the appropriate volume (200 µl) of transformed competent cells were spread onto plates containing agar, L Broth medium and the appropriate antibiotic (in this case, ampicillin at 50 µg ml⁻¹).

2.5.2. Restriction mapping of rescued plasmids

2.5.2.1. Purification and quantitation of Plasmid DNA

Mostly small scale "miniprep" plasmid DNA preparations performed by alkaline lysis method were used; rarely, large-scale preparations were used with the same method and proportionally larger amounts of solutions.

Single colonies were picked from a plate, made as described in section 2.3.2.4., and an overnight culture was grown for 16-20 hours at 37°C in 10ml L-broth medium containing the appropriate antibiotic (ampicillin at 50 µg ml⁻¹) with shaking. A bacterial pellet was produced from the desired amount of culture (1.5, 3, or 4.5 ml) by spinning at 13 000 g in a Microcentaur microcentrifuge, and cells resuspended by pipetting up and down gently in 100 µl Solution I (25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 50 mM glucose) and incubated at room temperature for five minutes. 200 µl of freshly prepared Solution II (0.2 N NaOH, 1% SDS) was then added, mixed by inverting the tube five times and stored on ice. Afterwards, 150 µl of ice-cold Solution III (5 M KOAc pH 4.8) was added and inverted several times, stored on ice 5-10 minutes and centrifuged at top speed for 10 minutes in a microfuge and the supernatant was transferred to a new tube. Following an equal volume of phenol:chloroform extraction (100 µl) the supernatant was transferred to a fresh tube again and after incubation for 20 minutes or more at -20°C the double stranded DNA was precipitated with 2.5 volumes of 100% ethanol at room temperature, centrifuged, and the resulting pellet was washed with 70% ethanol, left to dry out and redissolved in 50 µl of TE (pH 8.0) buffer containing DNAase-free pancreatic RNAase (20 mg/ml).

The amount of double-stranded plasmid DNA was measured spectrophotometrically, taking readings at wavelengths of 260 nm and 280 nm; then the calculation was made according to the criterion that an OD of 1 at 260 nm corresponds to approximately 50 µg/ml for double stranded DNA, or 40 µg/ml for single stranded DNA and RNA (Sambrook *et al.*, 1989).

2.5.2.2. Setting up digestion of rescued plasmid DNA.

Rescued plasmids were analysed by single, double and triple digests as follows:

Aliquots of 5-10 μ l plasmid mini-prep DNA, corresponding to \sim 5 μ g plasmid DNA, were mixed with 2 μ l of the appropriate 10X buffer, and the solution volume made to 19 μ l. 1 μ l of each restriction enzyme was added, and the digests were incubated at 37°C for 1-3 hours.

2.5.2.3. Calculation of the sizes of rescued flanking DNA sequences by agarose gel electrophoresis

To resolve the sizes of genomic inserts, $1/5$ or $1/10$ of the digested plasmid DNA was loaded with a gel loading buffer into 0.8-1% (w/v) agarose gel in 1X TBE and run at an applied voltage of 2-10 V/cm. A 1 kb ladder (Gibco BRL) was used as a standard marker along with the samples. After electrophoresis, gels were visualised on UV transilluminator following either post-staining with ethidium bromide (EtBr) or adding it to the sample (0.5 μ g/ml), and pictures taken with a Polaroid camera loaded with 667-land film and fitted with a Kodak Wratten filter No. 23A. The insert sizes were calculated by comparison with the standards.

2.5.3. Subcloning of the digested fragments of genomic flanking sequence into pBS

2.5.3.1. Setting up single, double and triple digest of rescued flanking DNA

The rescued plasmid DNA was digested with different enzymes as single or multiple digests and resolved by agarose gel electrophoresis. The resulting band or bands corresponding to the insert fragments were excised from the gel with as little agarose as possible, put into a small microfuge tube and left in the freezer (-20°C) for at least 2 hours or overnight. Then a hole was made in the bottom of the tube with a syringe needle, the tubes was placed into the larger 1.5 ml eppendorfs and centrifuged for 10 minutes at 6500-8000 rpm to elute the DNA into the larger eppendorf. Yields from this procedure were typically 50 %.

2.5.3.2. Ligation of digested fragments into pBluescript

The digested DNA fragments were then ligated with the plasmids (pBluescript) for subcloning. Parallel ligation reactions were set up with different insert/vector proportions as follows:

Materials	1/2	1/3
Vector	0.5 µl	0.5 µl
Insert	1.0 µl	1.5 µl
Buffer	1.0 µl	1.0 µl
Enzyme	1.0 µl	1.0 µl
Distilled water	6.5 µl	6.0 µl
Total	10 µl	10 µl

2.5.3.3. Transformation into *E. coli*

After preparation of competent cells (section 2.4.2.3), transformation was carried out in the same way, with the exception that X-gal and IPTG were added to the agar L-Broth medium in the plates for easy selection of transformed colonies; these are expected to be white.

2.5.3.4. Preparation of transformed plasmid DNA with inserts

A single white colony grown on the plate containing agar L-Broth medium with IPTG, X-gal and ampicillin was picked with a sterile pipette tip or a toothpick and inoculated into 10 ml of L-Broth medium containing ampicillin, and overnight cultures were grown for 16-20 hours at 37°C with shaking. Then from the cultures, small-scale DNA preparations were prepared and used for Southern blotting with probes prepared from the target DNA fragment.

2.5.4. P1 Clones

For preparation of genomic DNA from P1 clones kindly recieved from S. Russell, a special protocol was followed (Russell, pers. comm.).

An overnight culture was grown in 25 µg/ml kanamycin, 100 ml of L-Broth containing 25 µg/ml kanamycin was inoculated with 0.1 ml of the overnight culture, shaken at 325 rpm at 37°C to an OD₅₅₀ = 0.15, and 1 ml of filter-sterilized 0.1 M IPTG (0.6 g IPTG dissolved in 25 ml double-distilled water) was added to the 100 ml culture. The culture was grown for a further 3 hours at 37°C to an OD₅₅₀ = 1.3-1.5, then the cells were harvested in a GSA rotor for 10 minutes, and "maxi" prep DNA preparation was performed using the alkali lysis method (Sambrook *et al.*, 1989). Finally, the DNA was resuspended in a suitable volume of TE buffer.

2.5.5. Southern blotting

2.5.5.1. Labelling of probes by random priming

Following the purification of a digested fragment from the agarose gel, by cutting the desired band out as described in section 2.4.4.1., fragments of DNA to be labelled were denatured by heating to 99°C in a PCR machine for 10 minutes and cooled to -20°C immediately by quenching on dry ice. Then in a small microfuge tube the following were combined:

10 µl of distilled water, 2 µl of 10x hexanucleotide mixture, 2 µl DIG DNA labelling mixture, 5 µl of insert DNA (denatured), 1 µl Klenow fragment (final volume of 20 µl), and incubated at 37°C in a water bath for up to 20 hours. The labelled probes were kept in the freezer until needed.

2.5.5.2. Southern blotting

After agarose/1xTBE gel electrophoresis of the DNA to be hybridised with the probe, it was transferred onto Hybond N membrane according to the procedure of Southern (1975). Prior to the transfer, the gel was immersed in two 20 min changes of denaturing solution (0.5 N NaOH, 1.5 M NaCl), then neutralised in two 20 min changes of neutralising solution (0.5 M Tris-HCl pH 7.5, 3 M NaCl), on a "rock and roller" rotatory mixer and transferred to the membrane overnight using 20xSSC. DNA was then fixed to the membrane by exposing to 1200 µJ cm⁻² UV irradiation in a crosslinker (Spectralinker or Stratagene).

Filters were prehybridised in prehybridisation solution (750mM NaCl, 75 mM Na-citrate pH 7.0, 0.1% N-lauroylsarcosine, 0.02% sodium dodecyl sulfate (SDS), with 1.0% (w/v) of either "blocking reagent for nucleic acid hybridisation" (Boehringer) or alternatively Marvel™milk powder (Safeway) for at least 1 hour at 65°C in the hybridisation tubes in a Techne "rotisserie" oven.

The probe was denatured by heating in a boiling water bath for 5-10 minutes to denature the DNA, chilled immediately on ice, and added to the hybridisation solution. The prehybridisation solution in the tube was discarded and the hybridisation solution containing the DIG-labeled probe was replaced and the probe allowed to hybridise from 10 hours to overnight at 65°C. At the end of the hybridisation, the hybridisation

solution containing unannealed DIG-labeled probe was decanted and saved for reuse. The membrane was washed twice taking 5 minutes per wash in 2X wash solution (2X SSC containing 0.1% SDS) at room temperature (considered a low stringency wash); and twice for 15 minutes in preheated 0.1X wash solution (0.1X SSC containing 0.1% SDS) at 65°C as a high stringency wash.

For visualisation, detection was performed with the colorimetric detection reagents NBT and X-phosphate by the following method, performing all incubations at room temperature with shaking. First, the membrane was equilibrated in (filtered) buffer 1 (100 mM maleic acid, 150 mM NaCl pH 7.5 at 20°C) for 1-2 minutes. Then the filter was blocked in buffer 2 (1% (w/v) Blocking Reagent or milk powder dissolved in buffer 1), for 30 minutes. At the end of incubation, buffer 2 was poured off and the antibody (Anti-DIG-alkaline phosphatase) solution in 1:5.000 dilution in buffer 2 added, and the membrane incubated for 15-30 minutes. Following two washes in buffer 1 for 10 minutes each to remove unattached antibody, the membrane was equilibrated in buffer 3 (100 mM Tris-HCl, pH 9.5; 100 mM NaCl, 50 mM MgCl₂). Finally, colour on the filter was developed using the colorimetric detection reagents; a freshly-prepared mixture of 45 µl of NBT and 35 µl of X-phosphate in 10-15 ml of buffer 3 was added to the filter in a polythene bag sealed on three sides, and then the fourth side was sealed, and the bag placed in a dark drawer, and the reaction was allowed to proceed for up to 20 hours. At the end of the development, the reaction was stopped by washing the filter at room temperature in buffer 1 for 4 minutes, and the result documented by either photocopying or photographing.

2.5.6. Sequencing and sequence analysis

Double stranded sequencing reaction using the dideoxy chain termination method (Sanger *et al.*, 1977) was performed with Sequenase™ kit as described in the Sequenase Version 2.0 DNA sequencing kit or Sequenase Quick-Denature Plasmid Sequencing kit manuals (United States Biochemical Corporation).

Denaturing 6% polyacrylamide gel electrophoresis was performed in a Sequi-gen (Bio-Rad) apparatus, according to the manufacturer's manual.

Prior to loading in groups of 4, the samples were prepared by heating to 80°C in a thermal cycler for 5 minutes, snap-chilled on ice immediately, and the gel was run at 50°C for 2-6 hours.

Afterwards, the gel was dried for 2 hours at 80°C onto 3MM Whatman paper under vacuum, put down overnight with X-ray film in a cassette, and autoradiography was carried out at room temperature.

The DNA sequences read were input the computer using “Speakquencer” and “DNA Strider 1.2” programmes. Then they were compared with all known Genbank sequences using the Blast programme, accessed via the www with “Netscape”. Comparisons were made both to DNA (blastn) and deduced amino acid (blastx) databases; the former to detect whether closely similar DNA sequences were already known; the latter, to see whether the rescued plasmid contained a reading frame with similarity to a known peptide sequence.

2.5.7. *In situ* hybridisation to polytene chromosomes

2.5.67.1. Preparation of chromosomal squashes and pretreatment for hybridisation

Squashes were prepared essentially as described by Ashburner (1989). Larvae were grown at 18°C up to the third instar larval stage, washed in 0.7% NaCl solution, and after dissection in 45% glacial acetic acid on a clean slide, the glands were transferred onto a drop (10 µl) of 1:2:3 fix solution (1 part lactic acid: 2 parts acetic acid: 3 parts water) on a siliconised coverslip (18 mm²) and fixed for 5 minutes. It was covered by a clean subbed slide, the coverslip was tapped with a pencil for around 1 minute to spread out the chromosomes properly. After the slides were left at 4°C for one week, they were placed on dry ice for ten minutes, and the coverslips flipped off with a razor blade and immersed straightaway into freshly made ethanol:acetic acid (3:1) for ten minutes. Following dehydration in 100% ethanol for ten minutes and drying, slides were examined under a phase contrast microscope and those with well-spread chromosomes were chosen for hybridisation.

Prior to hybridisation the selected slides were incubated in preheated 2X SSC for 30 minutes at 65°C, washed in 2X SSC for two minutes at room temperature and then acetylated in 0.1 M Triethanolamine-HCl (pH 8.0)

and 0.125% acetic anhydride for ten minutes. After washing twice in 2X SSC for two minutes, slides were dehydrated twice in 70% ethanol and twice in 95% ethanol for 5 minutes and allowed to air dry. Afterwards, the denaturing step was carried out in freshly prepared 0.07 N NaOH for exactly 3 minutes, washed in 2X SSC for 5 minutes, and dehydrated in 70% and 95% ethanol again for 5 minutes respectively.

2.5.7.2. Preparation of nick-translated biotin probe

In order make biotin labelled probes for *in situ* hybridisation, 0.5 mg of plasmid (pBluescript) DNA, 2.5 ml of 10X nick translation buffer [0.5 M Tris-HCl pH 7.5, 0.1 M MgSO₄, 1 mM dithiothreitol, 500 mg/ml bovine serum albumin (BSA Pentax Fraction V)], 2.5 µl of dNTP mix (0.3 mM of each base), 2 µl of Bio-16-dUTP (Boehringer), 1 µl of ³²P (trace), 1.5 µl of DNAase I diluted 1:1000 in TM10 (10 mM Tris-HCl pH 7.5, 10 mM MgCl₂), made up to 25 µl with distilled water and 10 U of DNA Polymerase I, were mixed in an eppendorf. Following incubation at room temperature for 60 minutes, the probe was separated by adding 1 ml of 0.2 M spermidine and incubated on ice for 30 minutes. After centrifugation at 4°C for 10 minutes and the removal of the supernatant, the pellet was resuspended in 75 ml of hybridisation buffer (0.6 M NaCl, 50 mM Na-phosphate pH 6.8, 1x Denhardt's (0.02% BSA / 0.02% ficoll / 0.02% polyvinylpyrrolidone, 5 mM MgCl₂).

2.5.7.3. Hybridisation and signal detection

Prior to hybridisation, the biotinylated DNA probe was denatured by boiling for 3 minutes, and 20 µl applied to each slide and sealed with a 18X18 mm coverslip. After the edges of the coverslips were sealed with Cow Gum thinned with diethylether, the slides were incubated in a moist chamber at 58°C for 12-18 hours. At the end of incubation, Cow Gum and coverslips were peeled off in 2X SSC with forceps, the slides were washed 3 times in 2X SSC for 20 minutes each at 53°C.

For signal detection, the slides were washed twice in PBS for 5 minutes each, once in PBS-TX (PBS containing 0.1% Triton-X 100) for 2 minutes, rinsed in PBS at room temperature and 200 µl/slide of a mix : 1 drop of A + 1 drop of B (Vectastain Kit) in 2.5 ml of PBS was added. Slides were placed in a humid box, and the solution was distributed by placing a 22x40 mm coverslip on the slides, before incubation at 37°C for

45 minutes. At the end of incubation, after washing in PBS, PBS-TX and rinsing in PBS as before, slides were placed in a tray horizontally, 250-500 μ l of DAB solution (0.5 mg/ml DAB and 1/100 volume of 1% H₂O₂) was added to each slide and incubated in the dark at room temperature for 1 hour. Finally after washing in PBS and staining with 1:20 dilution of Giemsa in 10 mM Na-phosphate buffer (pH 6.8) for 12-20 minutes, the slides were washed in water and mounted with DPX mount or equivalent.

Chapter 3: Results

Enhancer trap expression patterns in the Malpighian tubules

This part of the thesis deals mainly with the following topics: The common P{GAL4} expression patterns in Malpighian tubules, mapping domains of gene expression of tubule sub-regions, mapping domains of gene expression of tubule cell types, quantification of tubule domains and cell types, reconciliation of genetic and functional maps, developmental aspects of stellate-cell specific GAL4 expressions, other results and preliminary molecular characterisation of the most tubule specific candidate genes.

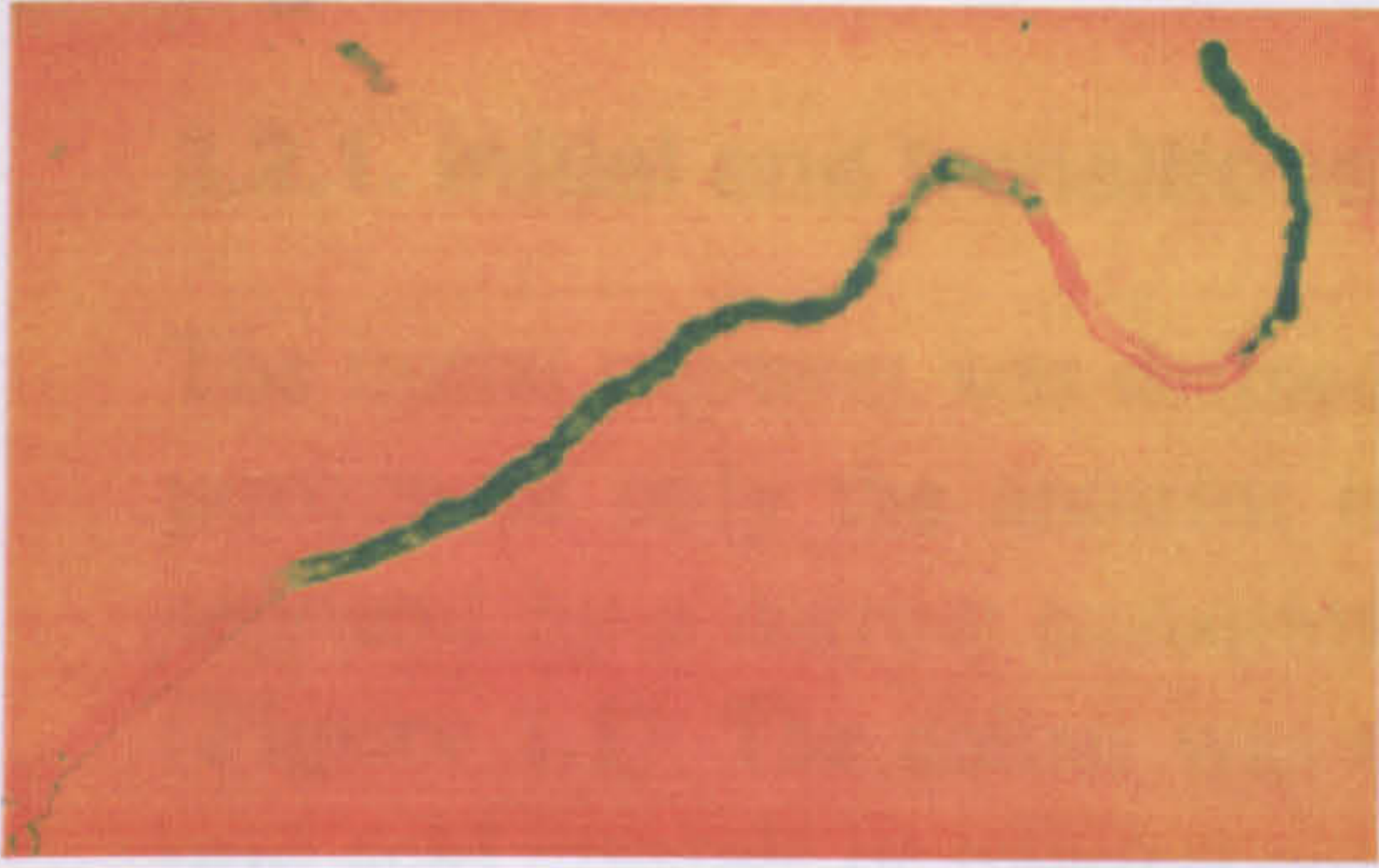
3.1. Common GAL4 expression patterns in Malpighian tubules

Of over 700 lines analysed by staining X-gal, the chromogenic substrate for β -galactosidase, in larvae or/and adults during the preliminary screening, around 150 show some staining in tubules. Of these, around 50 show region specificity (**Figs 3.1 a-c**) or cell-type specificity either principal (Type I) cells (**Figs 3.1 e-f**) or stellate (Type II) cells (**Figs 3.1 g-h**) or sometimes both region and cell type specificity (**Figs 3.1 d**). Of these, only 3 show expression confined strictly to tubules under all developmental stages analysed (**Figs 3.7-9**). Very few lines stain the whole length of the tubules. In those lines expressing β -galactosidase in a wide range of tissues, the most common expression pattern in tubules is a near ubiquitous staining, apart from the initial and transitional segment of the tubules (**Figs 3.1 i-j**).

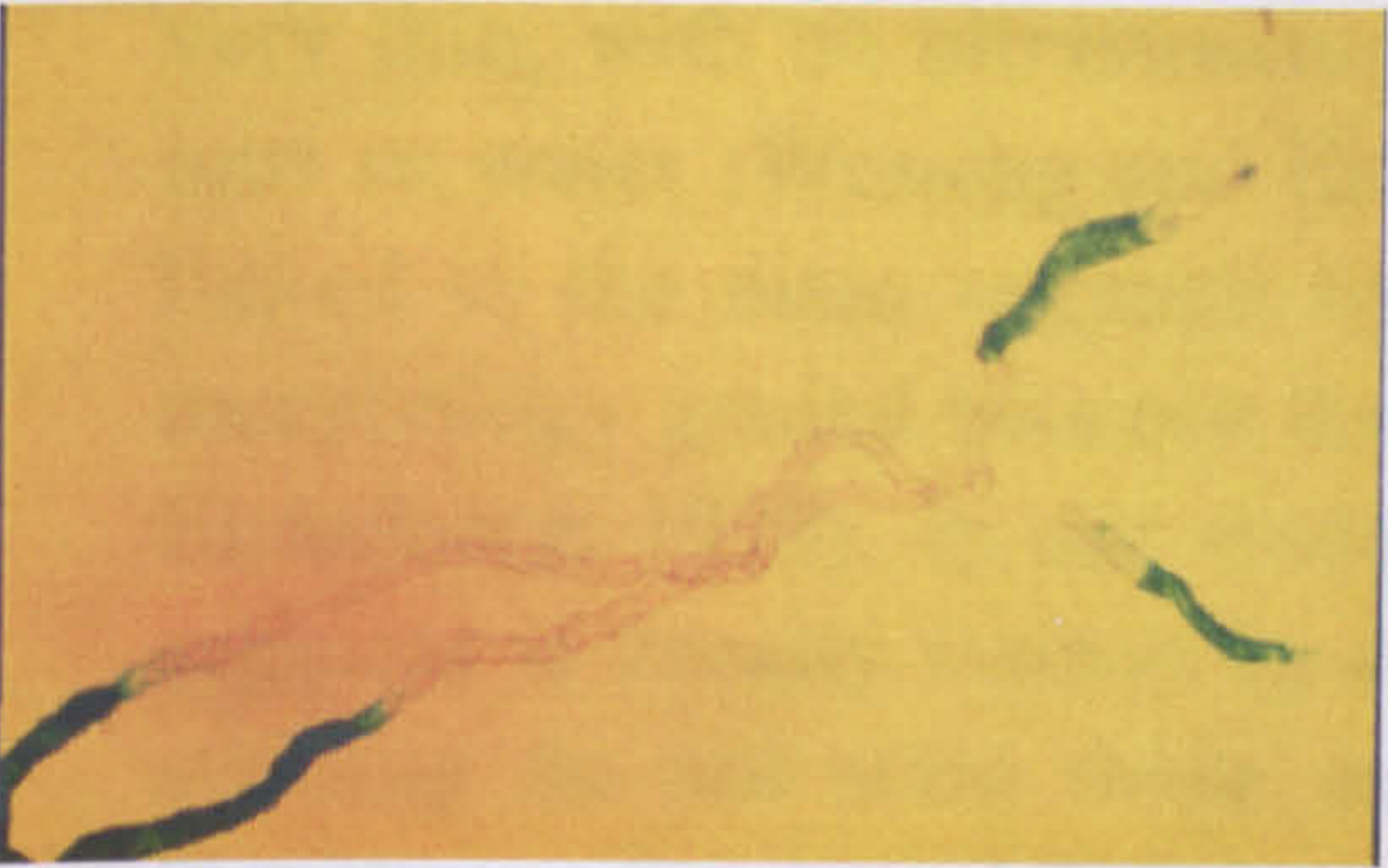
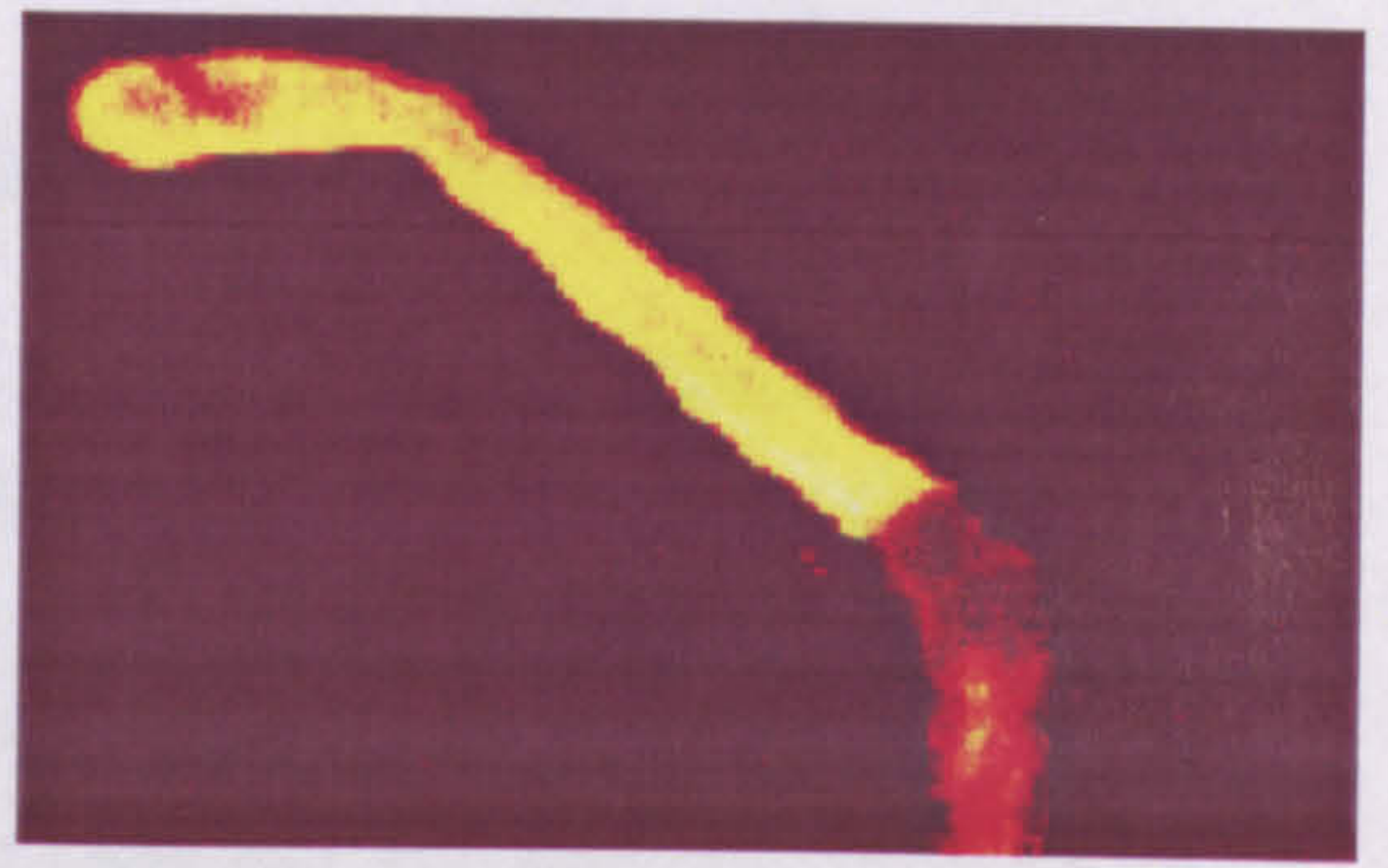
Figure 3.1. Common expression patterns in the Malpighian tubules observed during the preliminary screening in this study.

- a) In line C825, expression is in the main and initial, but not transitional or lower tubule, segments of anterior tubules.
 - b) In the same line, initial segment expression pattern is shown with fluorescein-coupled antibody staining against β -galactosidase.
 - c) In line C507, expression is in the transitional segment and lower tubule compartment of anterior tubules, a pattern complementary to the previous line.
 - d) Line 155Y labels the transitional segment of the anterior tubule.
 - e) In line C374, expression is in a subpopulation of principal (Type I) cells of main segment. Anterior tubules are shown here.
 - f) In line C801, expression is in a subpopulation of principal cells in the main segment of posterior tubule.
 - g) In line C724, expression is restricted to the stellate cells (Type II). Anterior tubules are shown here.
 - h) Posterior tubules of Line C724 also show staining restricted to the stellate cells.
 - i) The commonest expression pattern seen particularly in the anterior tubules in line C748. Note that there is also extensive staining of midgut.
 - j) The same expression pattern in line C303 at higher magnification.
-

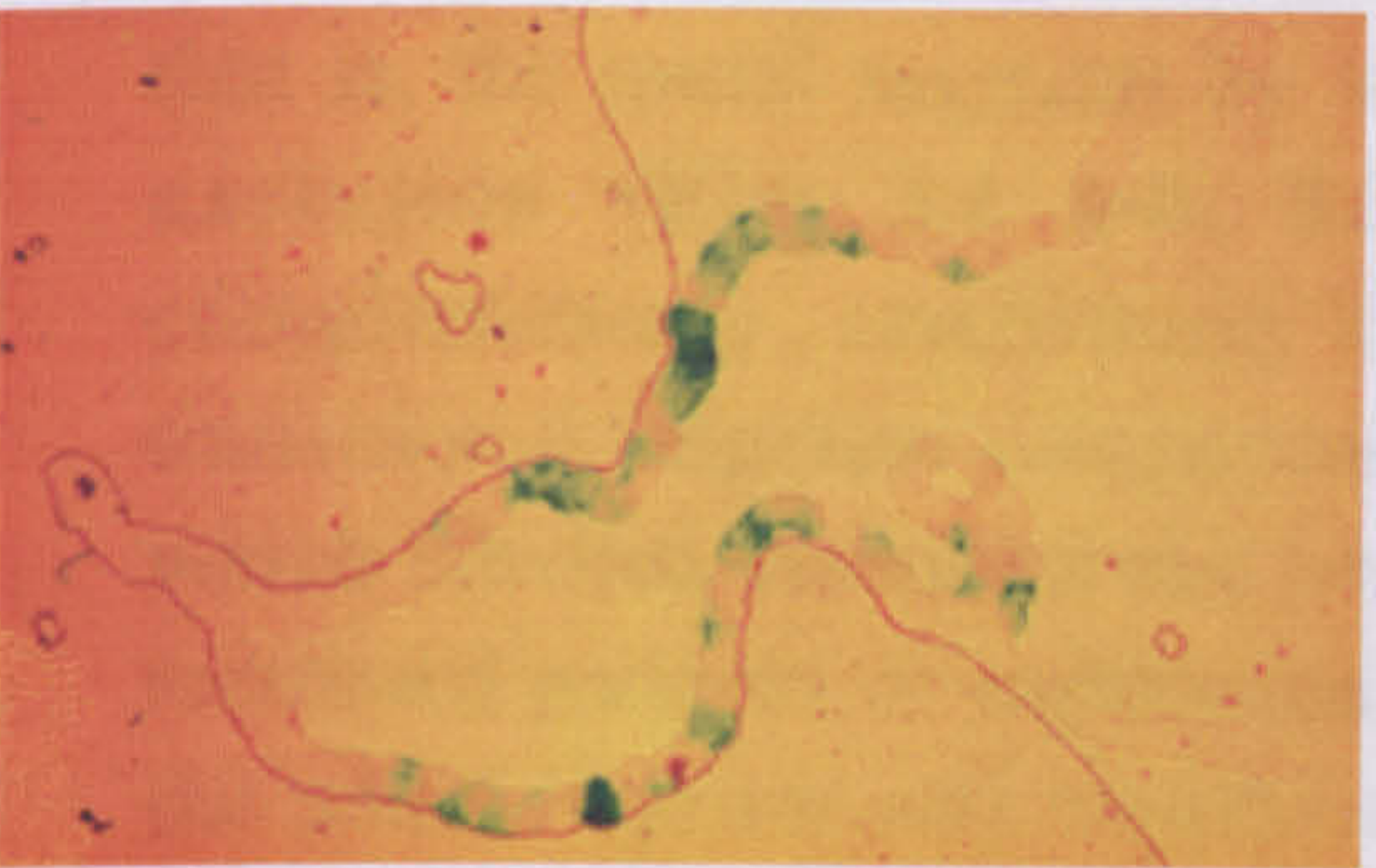
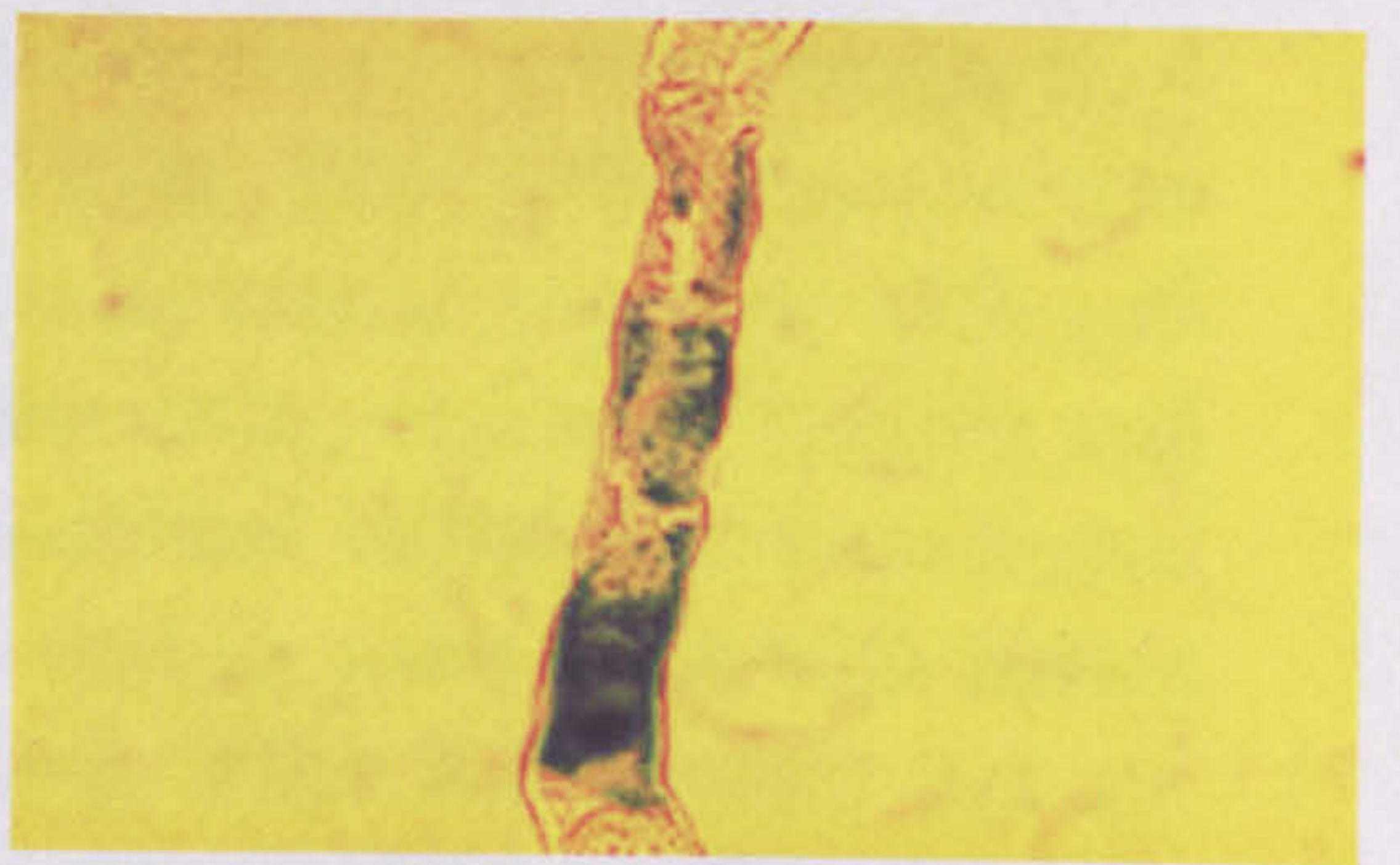
3.2. Mapping domains of gene expression of *Arabidopsis* regions



b)



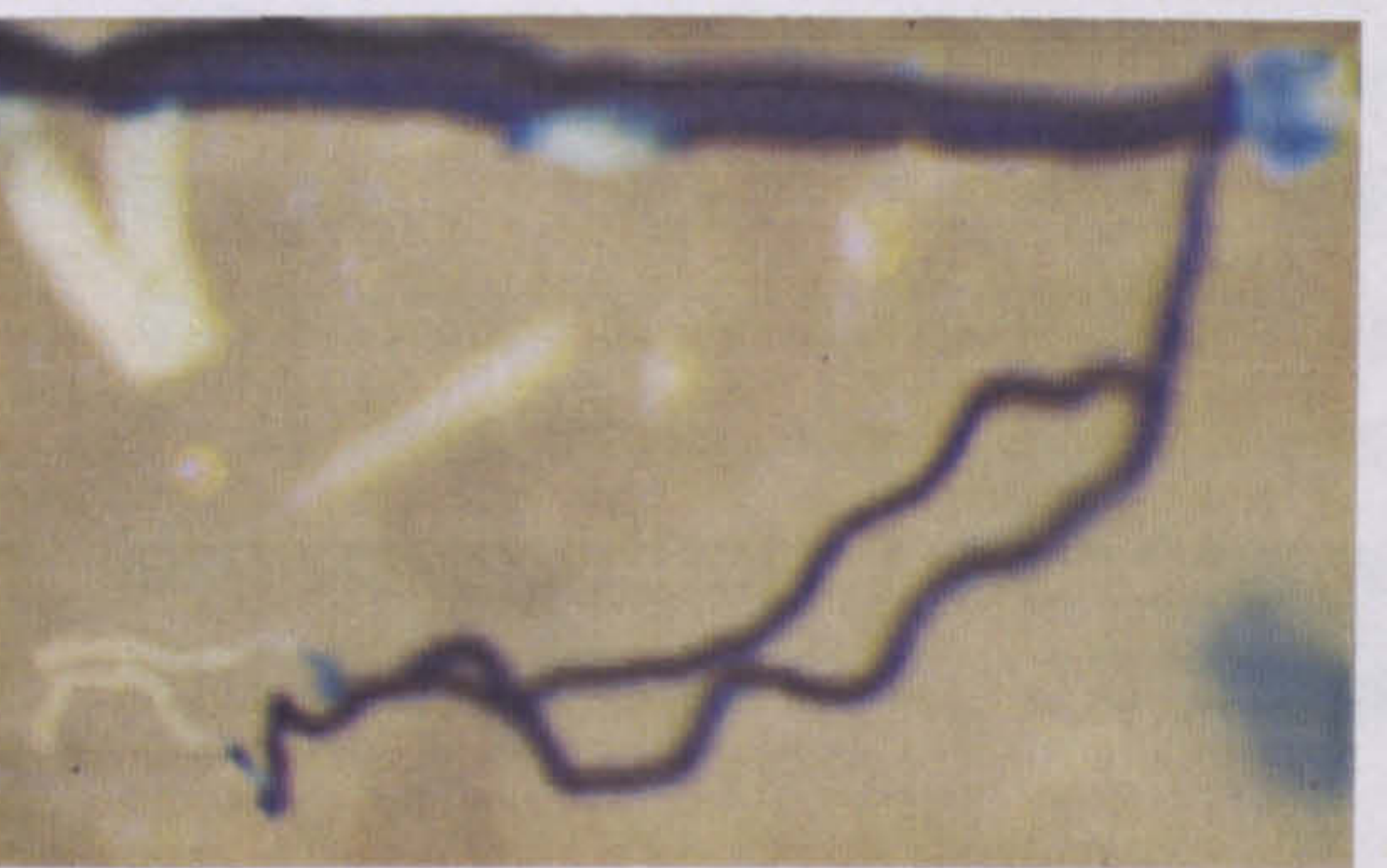
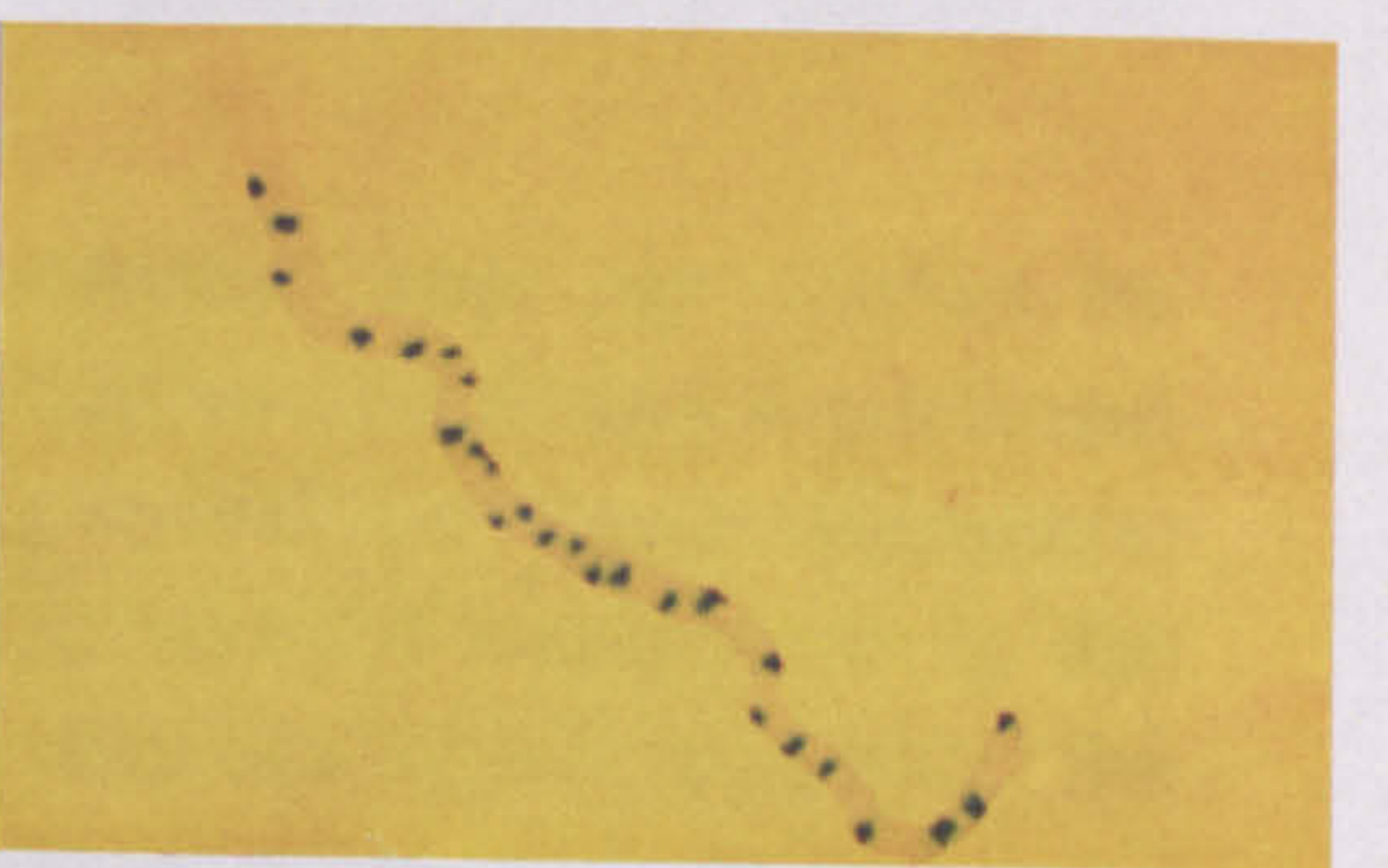
d)



f)



h)



j)



3.2. Mapping domains of gene expression of tubule sub-regions

3.2.1. Initial and transitional segments

The initial segment was defined classically as the white, distended distal portion of only the anterior tubules, linked to the transporting main segment by a narrow transitional segment of intermediate morphology (**Figure 1.1**). The colour was thought to be due to storage excretion of uric acid crystals or mineral concretions in the lumen, and the cells were very thin, with no ultrastructural specialisations for active transport of ions or water (Wessing and Eichelberg, 1978). The initial segment was linked to the main segment by a short transitional segment with cell morphology graded between the two neighbouring regions (Wessing and Eichelberg, 1978). In our studies, the combined initial and transitional segment is usually stained by exclusion; that is, most lines with tubule staining do not stain these segments (e.g. **Figs 3.1 i,j and 3.2 a**). However, a few lines label these segments specifically (e.g. **Figs 3.2 b-e**), that is, the initial segment by inclusion and the transitional segment by exclusion (**Figs 3.1 a-b, 3.2 c-e**). These boundaries are clearly complementary, confirming the genetic identity of the initial and transitional segment compartments (**Figure 3.2 a v. 3.2 b**). Those lines which labelled the initial and transitional segment tend to have relatively restricted patterns of expression elsewhere in the tubule, suggesting that the initial segment may express a relatively distinctive genetic repertoire.

3.2.2. Transitional segment

A transitional segment could also be inferred from our studies by comparison of the boundaries of lines staining initial and transitional segments compared with those staining initial and main segments. Although one (or two) lines (e.g. C507) showed expression in the transitional segment and in neither the main nor initial segments, the transitional segment was usually detected by an absence of staining. Line C825 stained the initial and main segments, but not the transitional segment (**Figure 3.2 c-e**); whereas line C507 appeared to stain the transitional segment but not the neighbouring regions (**Figs 3.1 c and 3.2 f**). In both cases, the scope of the transitional segment was consistent with that recorded classically (Wessing and Eichelberg, 1978). It is also clear that the stellate cells (type II cells of Wessing, *op. cit.*) of the transitional

segment are not included in the staining (**Figure 3.2 f**); so this line provides evidence of both region and cell-type specificity in the transitional segment.

One striking finding was that all those lines delineating the initial or transitional segment in anterior tubules also reported an analogous boundary in posterior tubules, although this had not previously been reported. In fact, **Figs 3.2 a and b** both show posterior tubule staining; the only difference between anterior and posterior tubules was in the relative length of the combined initial and transitional segments, at 44 and 7 cells respectively (see **Figs 3.2 c, d & Figure 3.5 a**). So it seems clear that posterior tubules are similar to anterior tubules in respect to gene expression, but merely with a miniature initial and transitional segments, which are perhaps too short to allow the accumulation of the insoluble white material originally used to classify the initial and transitional segments in anterior tubules.

3.2.3. Main segment

The main segment was defined as an apparently uniform epithelium containing cells with abundant mitochondria and microvilli (specialisations associated with active transport), extending from the transitional segment to the ureter (**Figure 1.1**). No staining pattern was found which is entirely specific to the main segment, although some stain some or all of the cells in the main segment. In **Figure 3.2 g**, the boundary between transitional and main segment is sharply marked by line C374; however, the main segment appears to taper off into the lower tubule.

3.2.4. Lower tubule

Firm evidence for a distinction between main segment and lower tubule, as distinct from ureter, can be found in lines C507 and C232; these mark a boundary at 30-40% of the distance from the junction with the gut (**Figure 3.2 h**). These lines stain the whole of the lower tubule, ureter and extend a short distance along the hindgut, though not the midgut.

3.2.5. Upper ureter

Although the staining of lines C507 and C232 demonstrates continuity between lower tubule and ureter (**Figure 3.2 h**), the classical scheme

(**Figure 1.1**) had placed a division between the main segment and ureter (Wessing and Eichelberg, 1978). However, the classical subdivision is matched by line C649, which marked a domain with a distal end precisely at the tubule bifurcation (**Figure 3.2 i**).

3.2.6. Lower ureter

There is also evidence that the ureter was in turn subdivisible into an upper and lower domains. Staining in the line C649 extends proximally from the bifurcation to a point midway along the ureter (**Figure 3.2 i**). This boundary is also marked by line c601 which labels the proximal portion of the ureter and posterior midgut (**Figure 3.2 j**). Incidentally, in common with most lines staining the upper ureter, staining includes the anterior hindgut posteriorly, but not the midgut anteriorly to the tubule insertions, presumably reflecting the relatively major distinction between ectodermal and endodermal origins of hindgut and midgut.

Figure 3.2. Mapping domains of gene expression of tubule sub-regions.

The pictures in this figure are obtained all from adult with an exception of picture 3.2 i which is from third instar larva.

a) Lines C709 (shown here) or C776 (not shown) mark the initial and transitional segments by exclusion.

b) Line 155Y marks the initial and transitional segments by inclusion.

c) Line C825 shows staining of the miniature initial and main, but not transitional, segment in posterior tubules (anti β -gal fluorescence).

d) As (c) but showing an anterior tubule, and thus showing the extents of the initial (stained) and transitional (unstained) segments (anti β -gal fluorescence).

e) Line C825 marks the transitional segment of anterior tubule by exclusion; histochemical staining for β -gal.

f) Line C507 marks the transitional segment by inclusion.

g) Line C374 marks the main segment. In this line the boundary with the transitional segment is precise, but the transition to lower tubule is gradual.

h) Line C507 marks the lower tubule and ureter; see also Figure 5. (Note that in this figure the second tubule from the top is broken, and so should not be used to gauge the relative size of the lower tubule).

i) Line C649 marks an “upper ureter” compartment from the tubule bifurcation to halfway along the ureter (anti β -gal fluorescence).

j) Line C601 marks the lower ureter, confirming the subdivision of the ureter of **Figure 3.6 f**. In this case, note that the staining extends to the hindgut, but does not extend anteriorly to the midgut, confirming the presence of a clear boundary of gene expression immediately anterior to the tubule insertion point.

3.3. Mapping domains of gene expression of muscle cell types

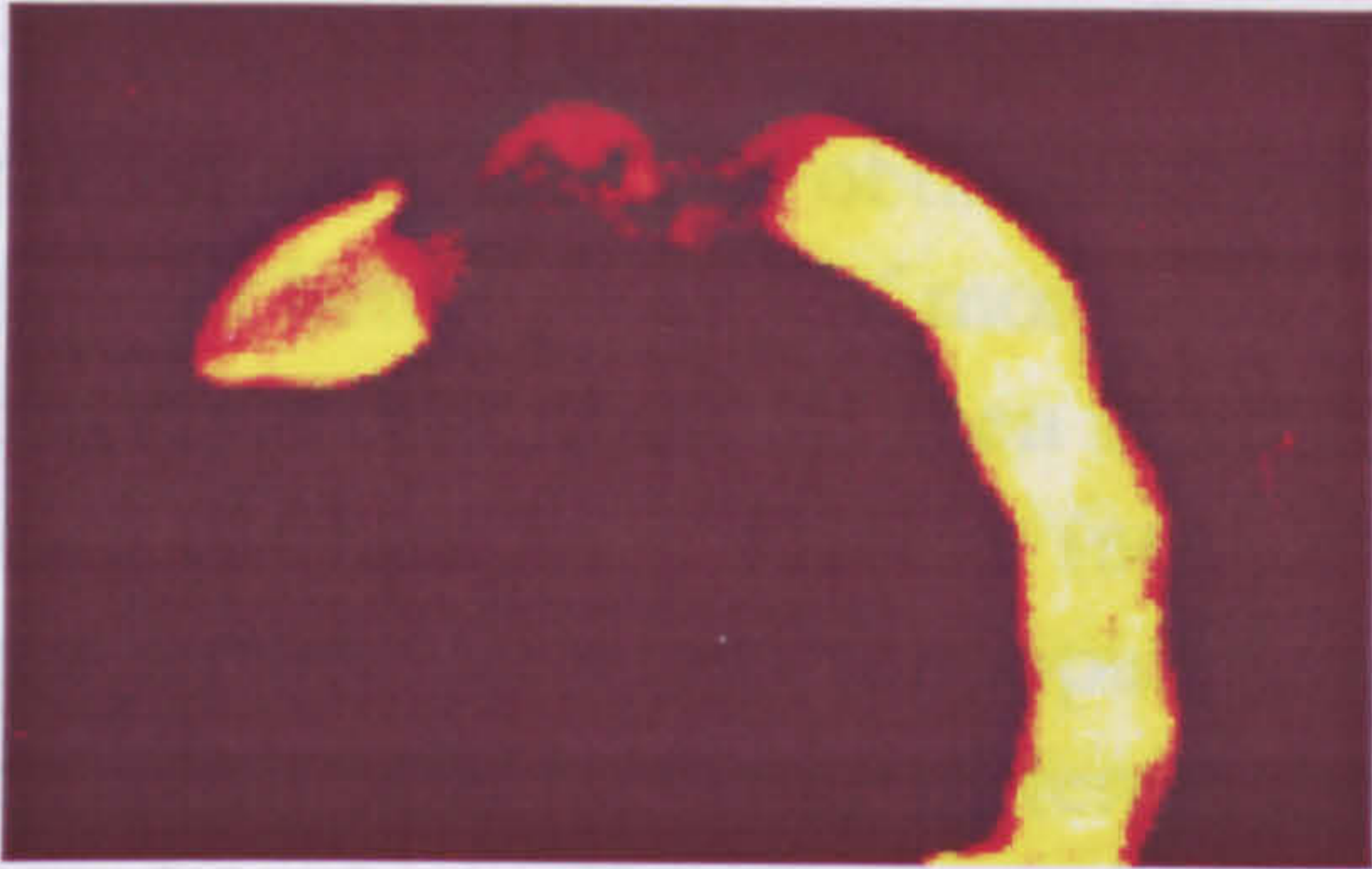
a)



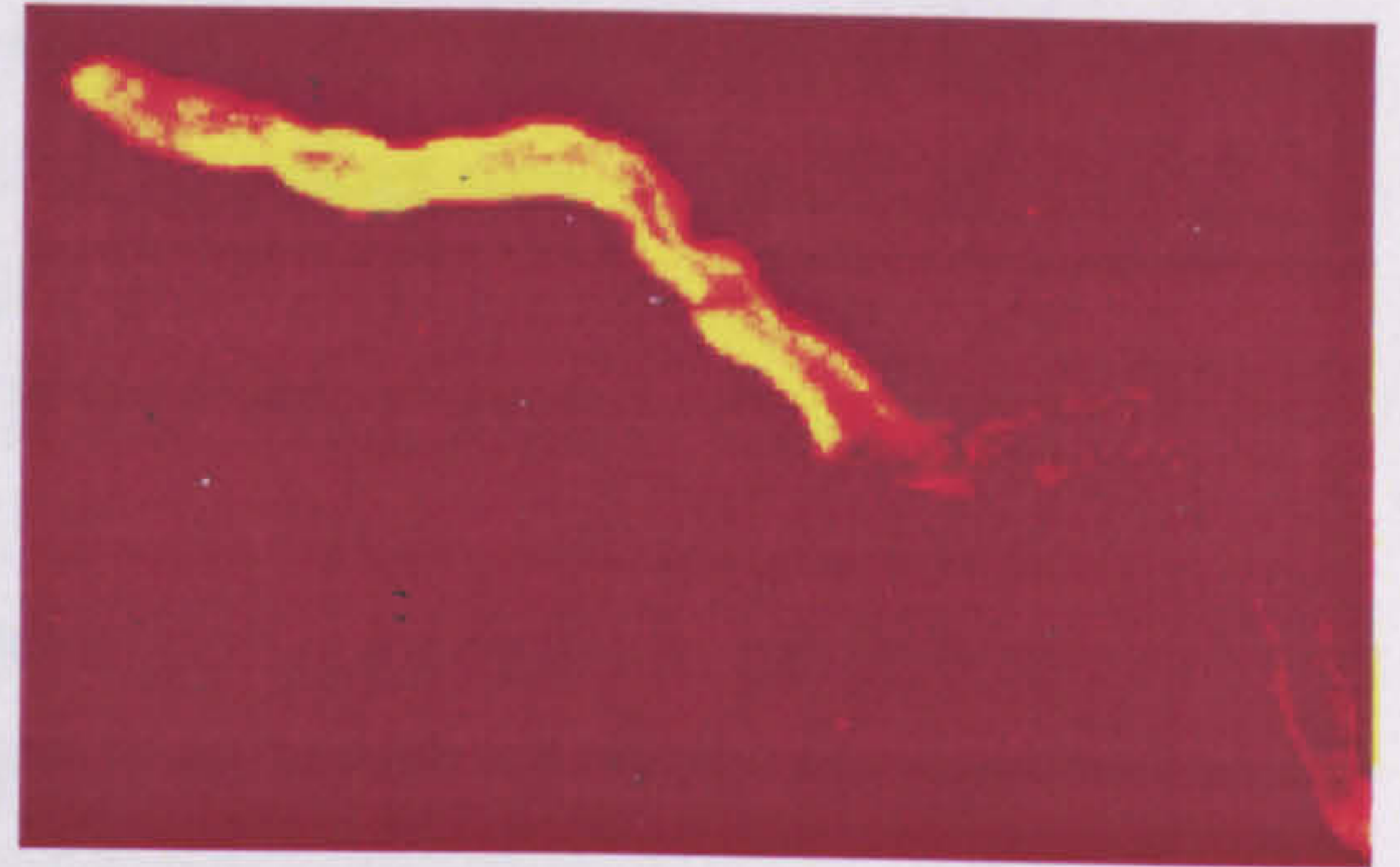
b)



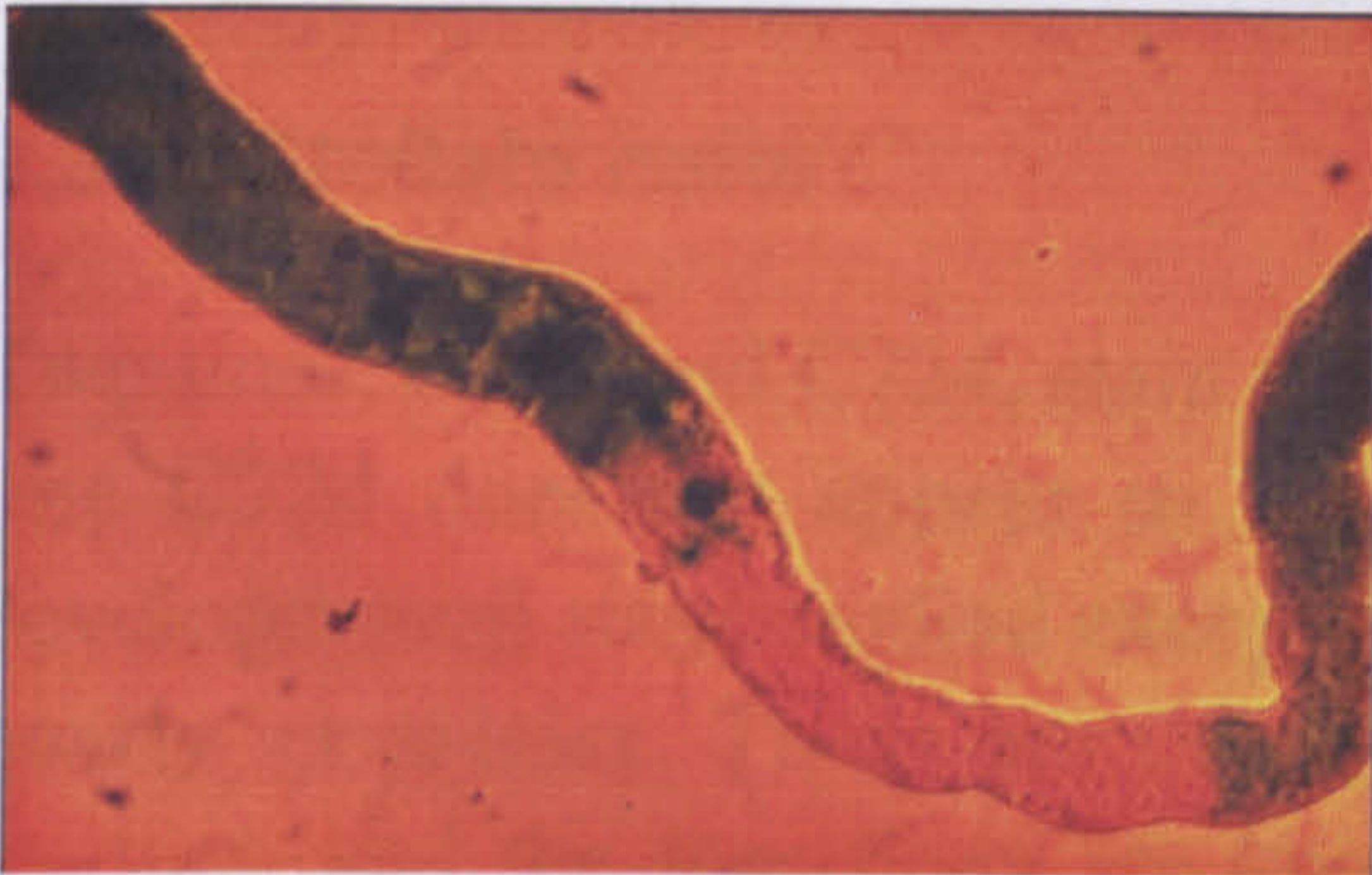
c)



d)



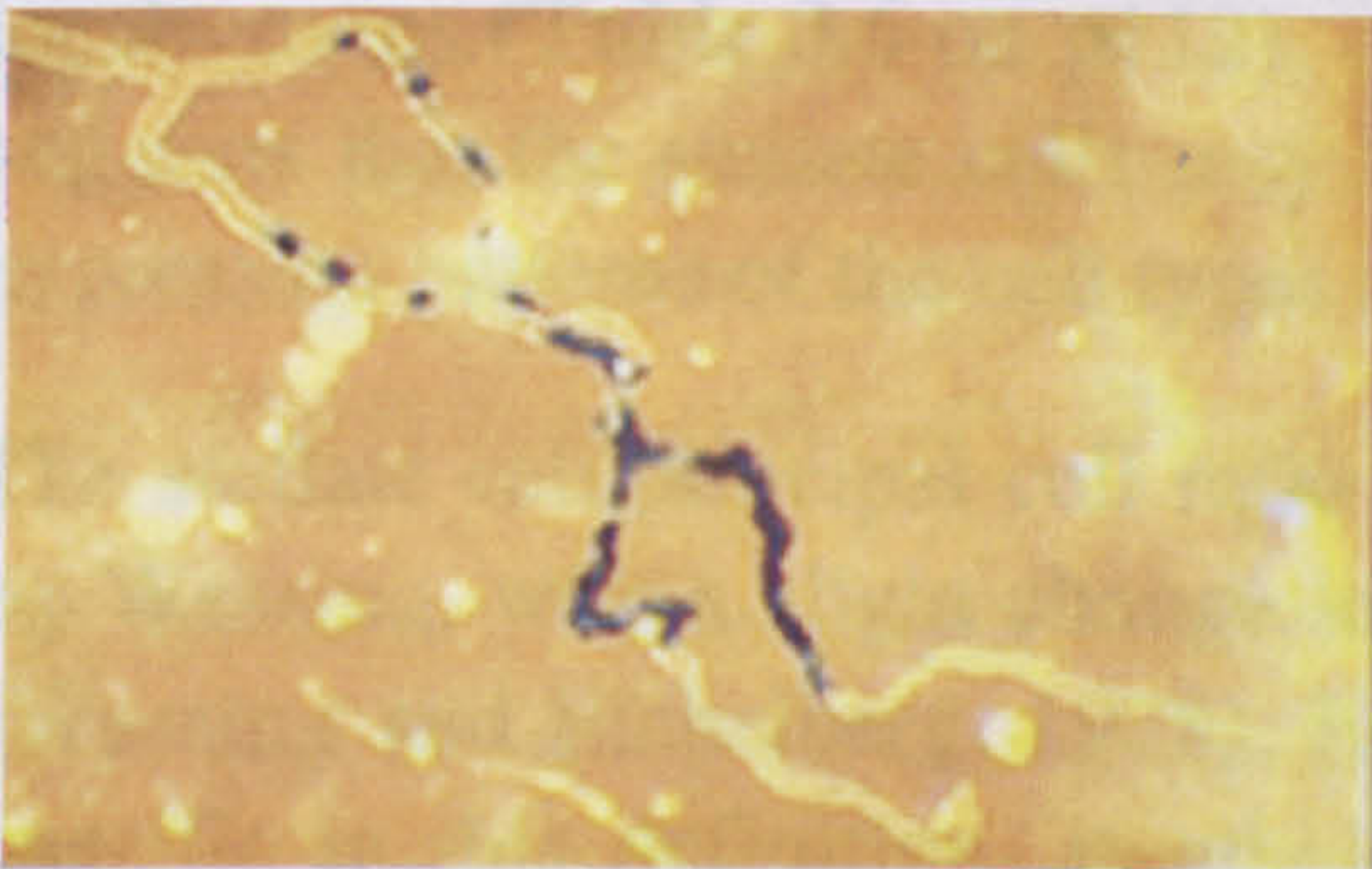
e)



f)



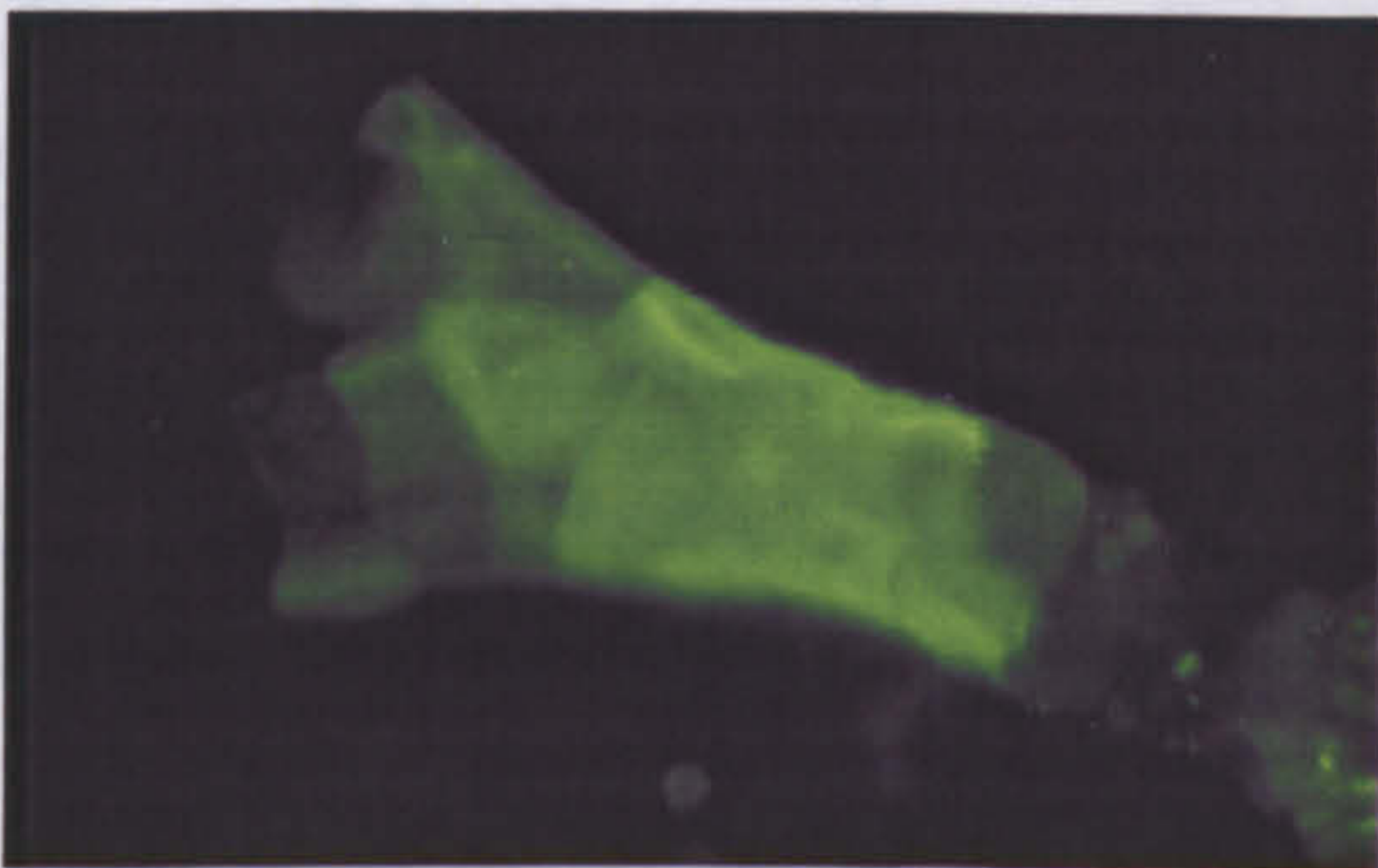
g)



h)



i)



j)



3.3. Mapping domains of gene expression of tubule cell types

3.3.1. Principal cells

Classically the tubule is known to be divided into principal (type I) and stellate (type II or intercalated) cells (Wessing and Eichelberg, 1978). However, it was found that the principal cells could be resolved into at least two distinct subpopulations, based on enhancer trap staining of line C324 (**Figure 3.3 a-b**). The exact fraction of cells labelled in this way varied from individual to individual, and it is not possible to discern any obvious trend in temperature-, sex- or age-specificity, although some preliminary data was obtained (Section 3.7.2) suggesting sex-specificity. Similar heterogeneity of morphologically-indistinguishable main segment principal cells could be seen in several lines (**Table 3.6**), and particularly in a distinct line, C568A (not shown). So, in at least some genetic properties, different subpopulations of otherwise apparently uniform principal cells are doing different things at any given time; in fact, *no* line was found which marked *all* the principal cells of the main segment.

3.3.2. Stellate cells

Two lines, C710 and C724, stain the stellate cells (**Figs 3.3 c-d**); of these, line C724 does not appear to stain any other cell in the fly at any developmental stage studied except in the early embryo, and C710 is very nearly as precise. These are the most tubule-specific lines of the 700 studied, and the insertions are known to have different chromosomal localisations (**Table 3.6** on p112), and thus to be in distinct genes. This suggests that the stellate cells may have the most unique subset of gene expression patterns of the cell types studied.

The stellate cell:principal cell ratio was described as approximately 1:2 by Wessing, with a slight change in ratio along the length of the tubule (Wessing and Eichelberg, 1978). Enhancer trapping allows to be more specific in this definition, as ethidium staining of nuclei can be used (**Figure 3.4**) to count those cells labelled as a fraction of total cells. This is uniformly 21-24% in all regions (initial, transitional and main) that have stellate cells (**Figure 3.5 a**). The occurrence of stellate cells respects the main/lower tubule boundary described above; the lower tubule does not have stellate cells, and the most proximal stellate cell in a tubule can be seen in **Figure 3.4 e**.

In the anterior tubules, both lines further marked bar-shaped cells in the initial and transitional segments, which can be taken to be counterparts of the stellate cells of the main segment (**Figs 3.3 c-d**); again, the ratio of stellate:principal cell is 1:4 (**Figure 3.5 a**). The transition between the two morphologies occurs at the proximal part of the transitional segment, with the first stellate cell sitting at the transitional/main segment boundary (arrowed in **Figure 3.25 a**). Given that neither line stains elsewhere in the animal, that the ratio of stellate : principal cells is similar throughout the length of the tubule, and that the change in morphology between bar-shaped and stellate occurs in the transitional segment without overlap, it can be considered that these bar-shaped cells are the stellate cells of the initial and transitional segment of the anterior tubules. In the posterior tubule, by contrast, the one or two stellate cells in the miniature initial and transitional segments are always of stellate morphology. However, despite their overall homology, a genetic difference between bar-shaped and stellate morphologies is reported by line C649, which stains uniquely bar-shaped cells (**Figure 3.3 e**).

A single cell at the tip of the anterior tubules was frequently labelled in these lines (**Figure 3.3 f**). Although these resemble the tip cells described by Skaer, which promote cell proliferation during embryogenesis, these are not thought to persist to adulthood (Hoch *et al.*, 1994; Skaer, 1989; Skaer and Martinez-Arias, 1992). Given that the function of several neurogenic genes is to produce an overdispersed pattern of sensory hairs which make up the PNS (Lawrence, 1993), the conspicuously even spacing of the stellate cells might also be generated by such signalling (**Figure 3.25 a and 3.1 g-h**). It should be noted, however, that the distribution of stellate cells is not perfectly uniform; on occasion, some cells appear to be very close (**Figure 3.4 a**) or even to touch (not shown).

In summary, although our results are broadly consistent with those of Wessing (Wessing and Eichelberg, 1978), enhancer trapping shows a highly predictable and consistent number of type II cells within each tubule segment, and with a generally even spacing, subject to minor local irregularities.

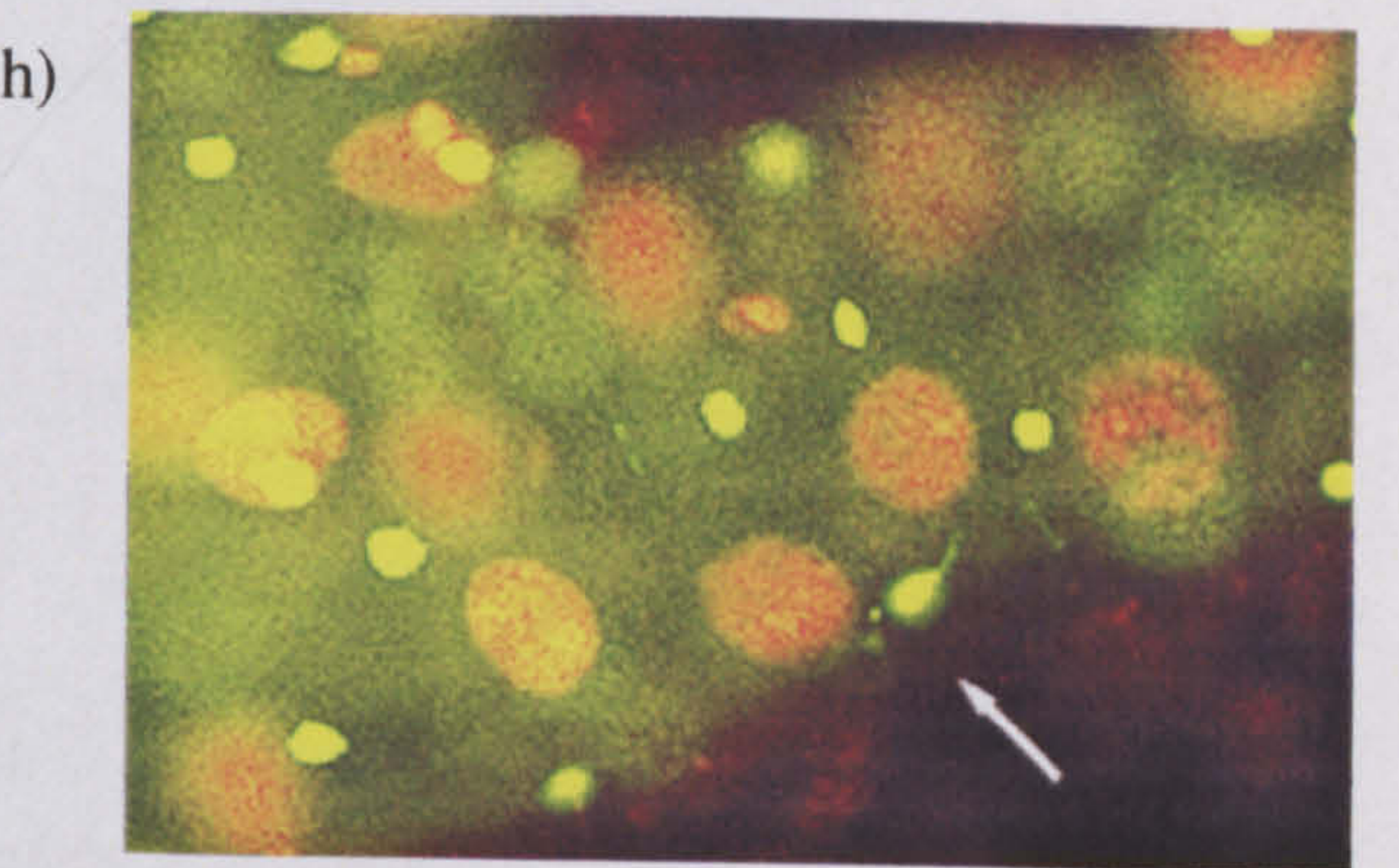
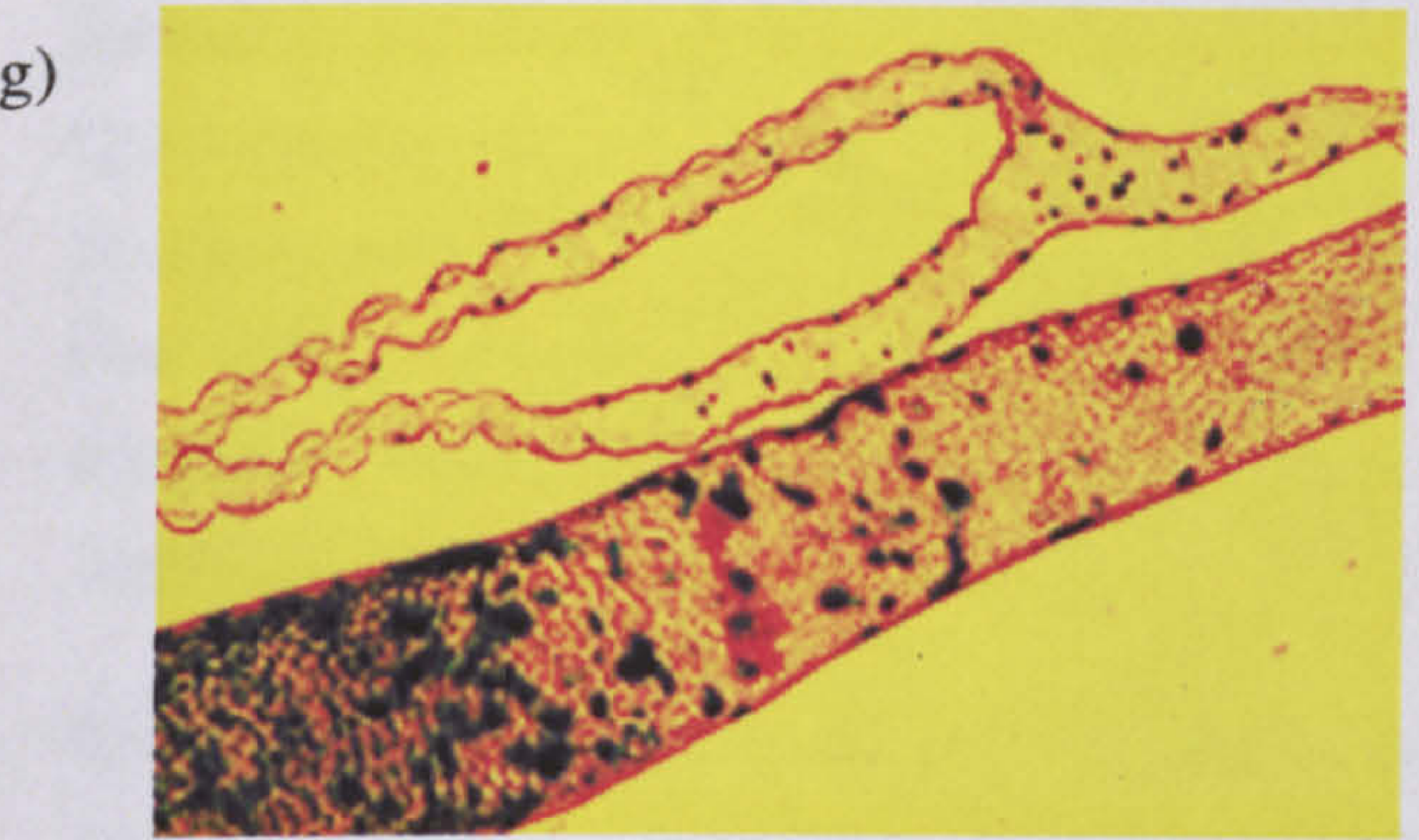
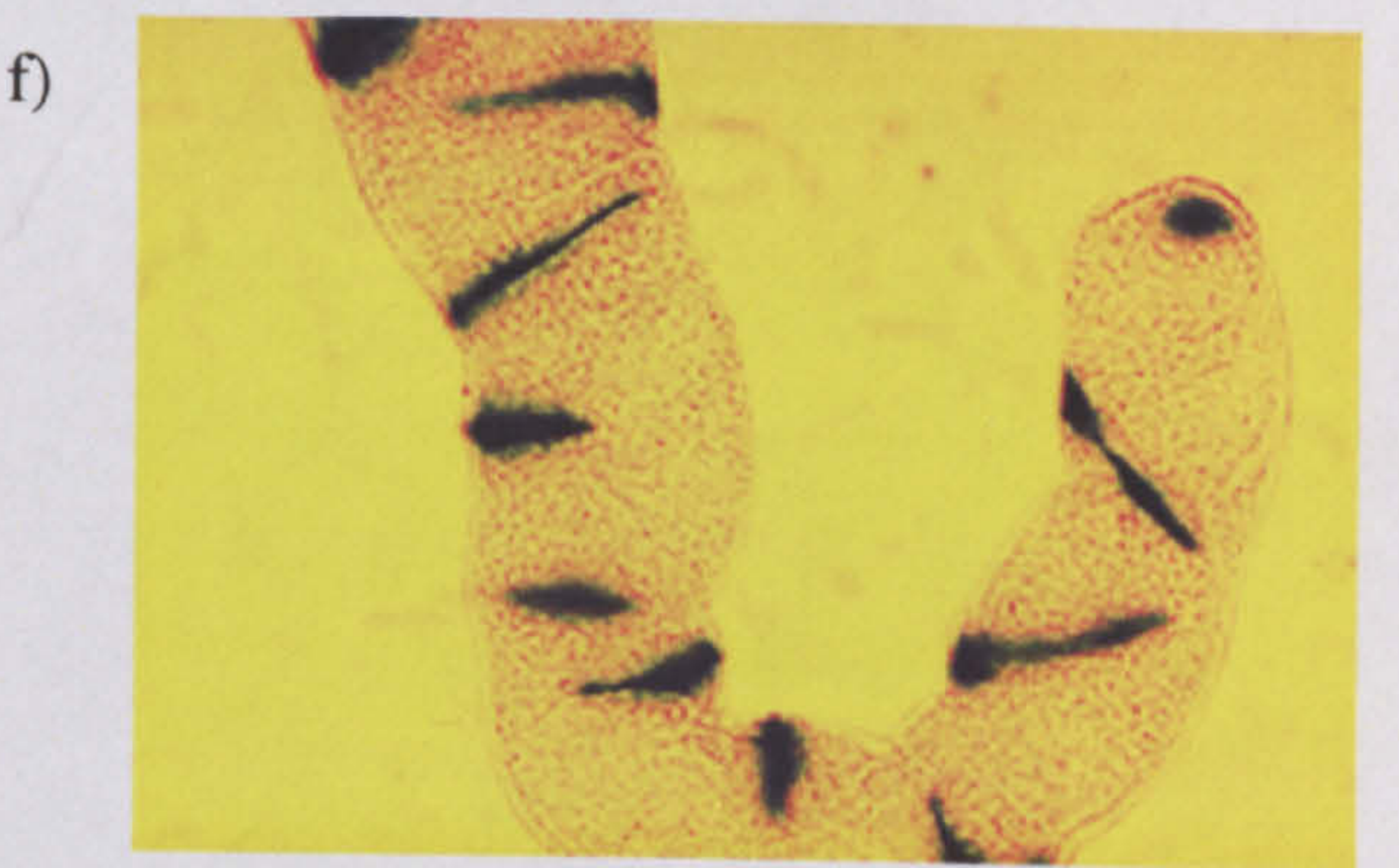
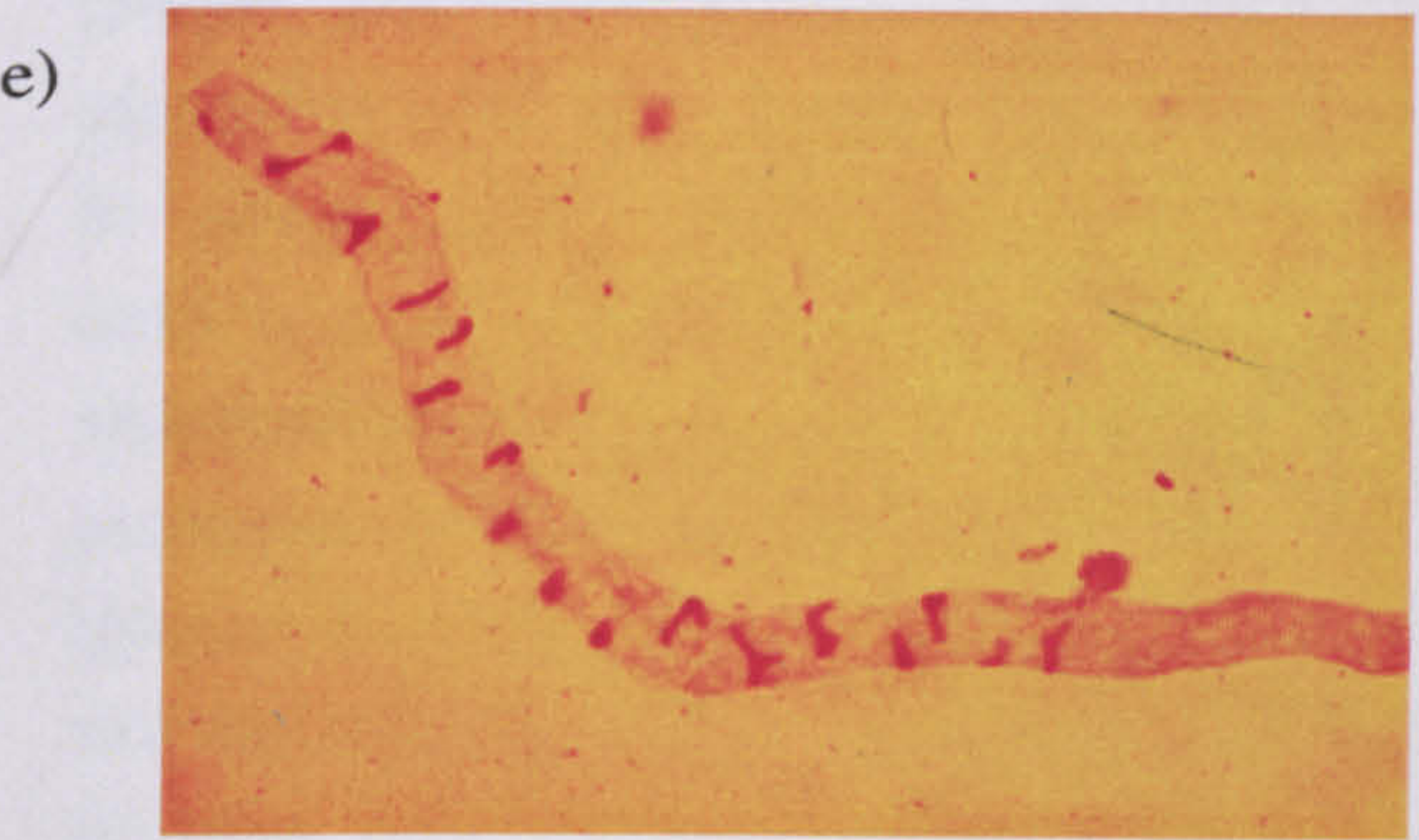
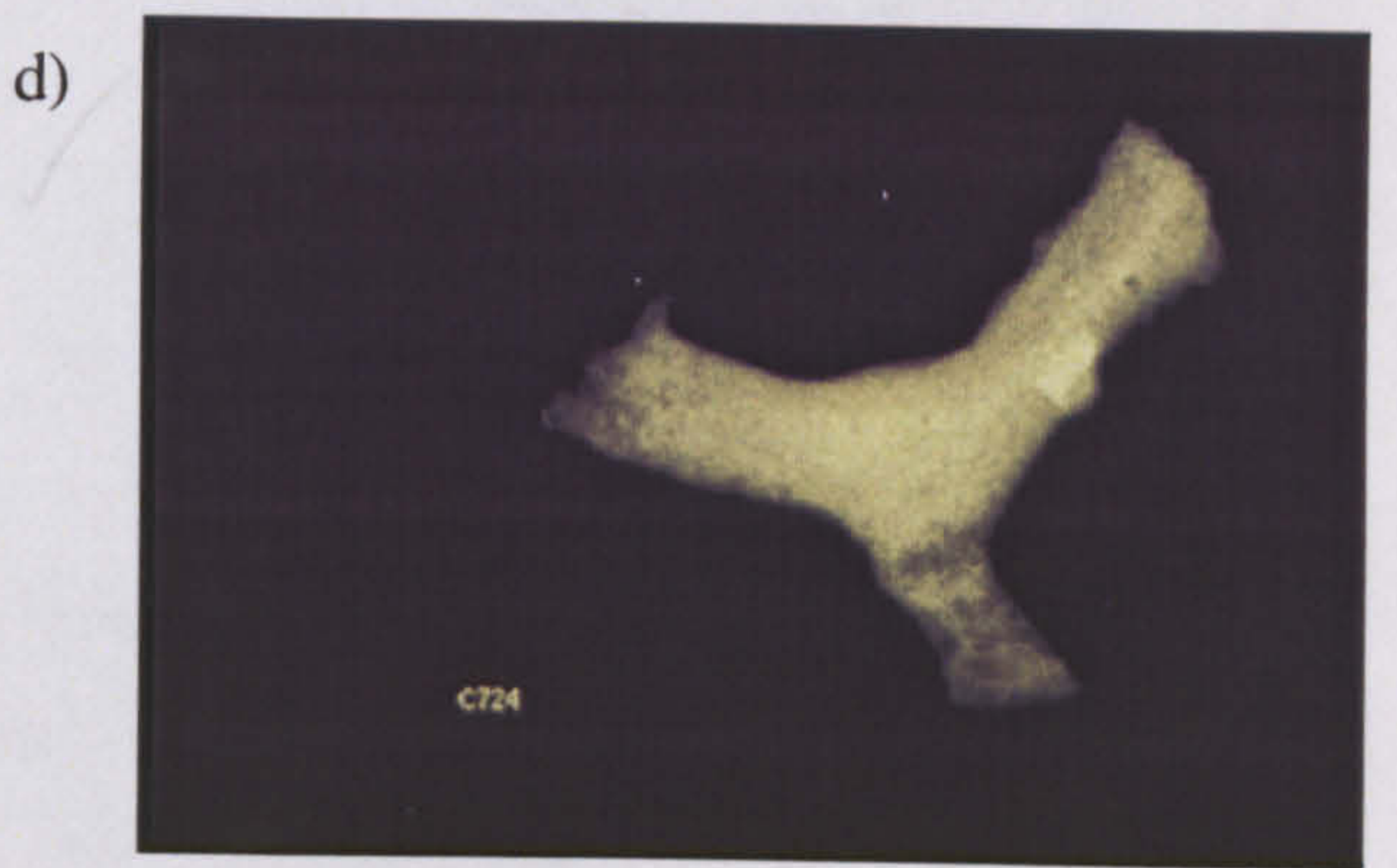
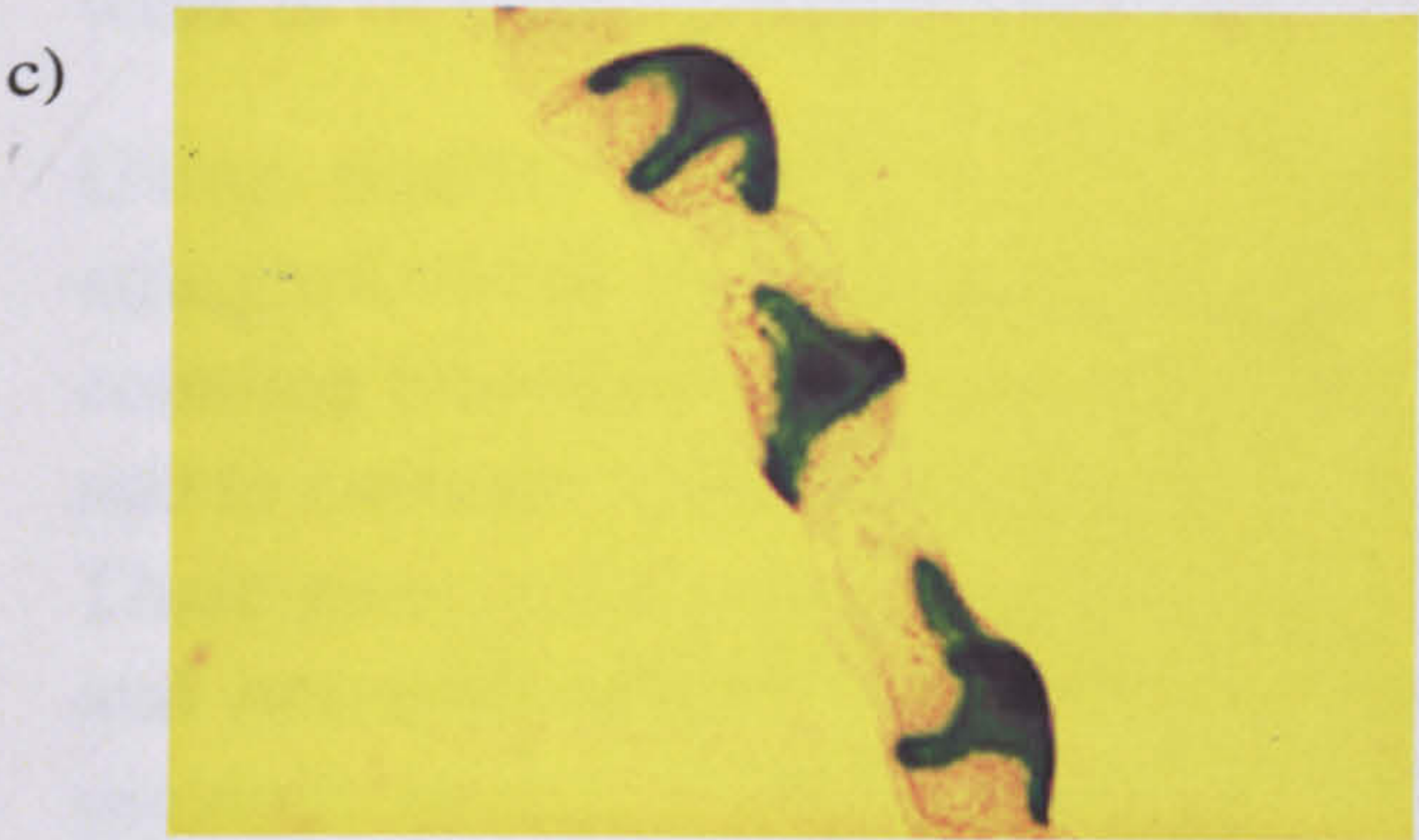
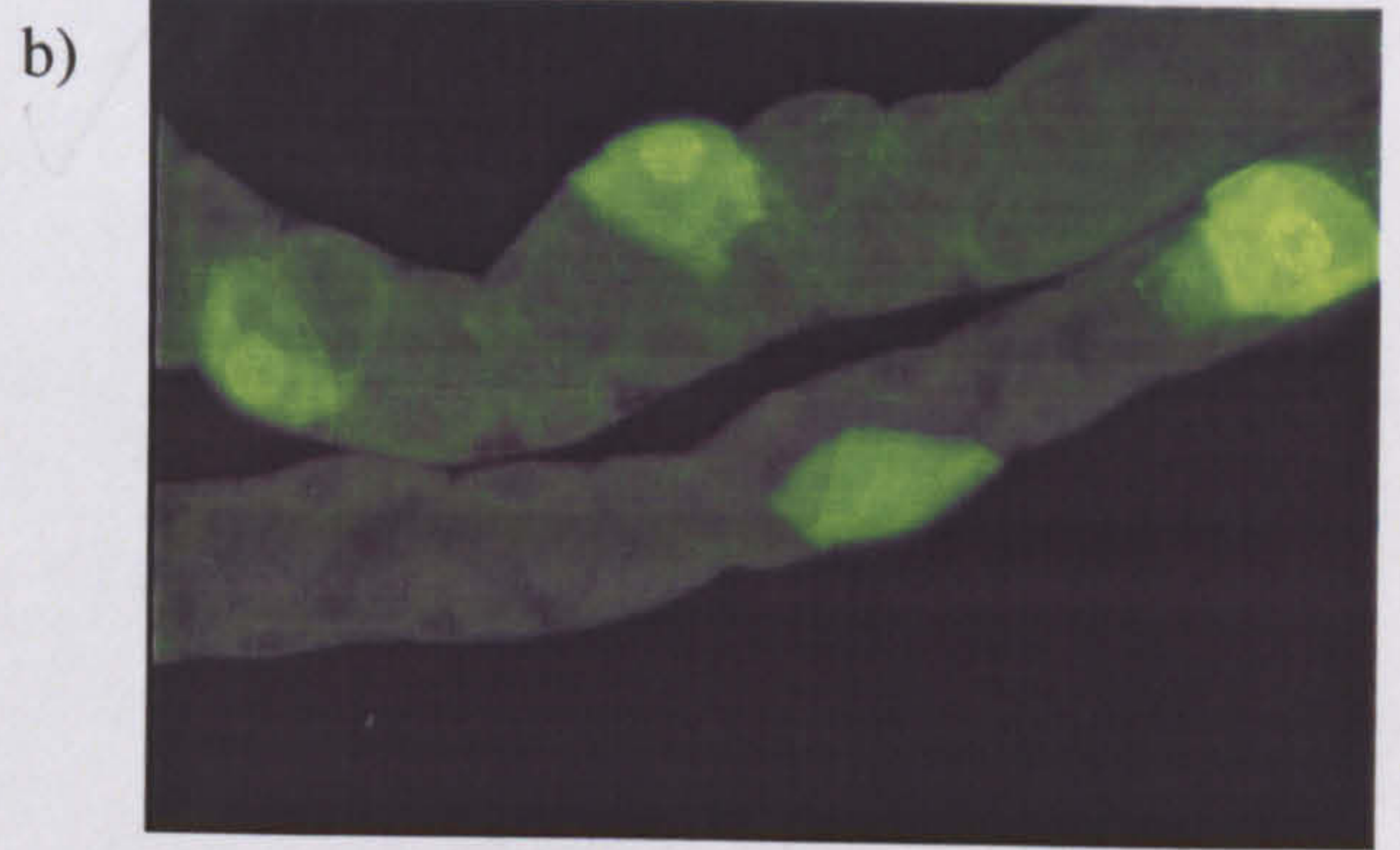
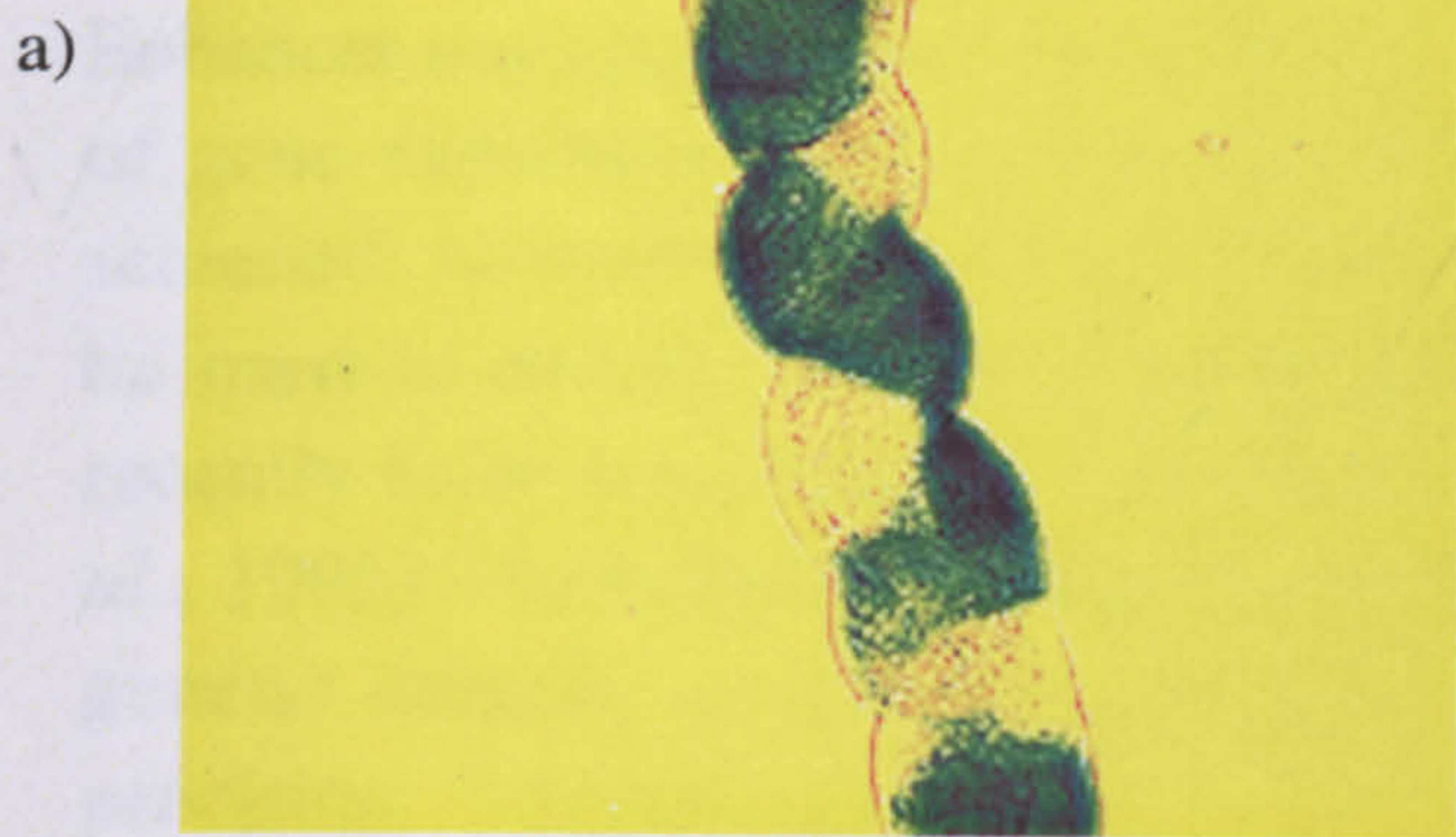
3.3.3. Tiny cells

Interestingly, several lines reported expression in a novel cell type found in lower tubules and posterior midgut (**Figs 3.3 g-h**). These cells contain

a reduced cytoplasm and a compact nucleus, and are only around 3-5 μm in diameter. Tiny cells are never seen in the same genetic domain as stellate cells (**Figure 3.4 e**), and the higher spatial resolution obtained using anti- β -gal immunofluorescence frequently shows thin processes (**Figure 3.3 h**). As far as is known, such cells have not previously been described in *D. melanogaster*; possibly they are counterparts of the myoendocrine cells recently described in *Formica* (Garayoa *et al.*, 1994). There are two likely candidates for such cells in some insect epithelia; both regenerative cells and neuroendocrine cells have been described. Regenerative cells usually occur in “nests”, are basally located and triangular in shape. The tiny cells are single, round with a neurite-like process, and found only in the lower tubule. Further, regenerative cells are unlikely to play a physiological role in adult tubules, as the cell number is fixed during embryonic development, with further size increase accomplished by an increase in ploidy without further cell division. The speculation (discussed later) is that tiny cells may prove to be neuroendocrine, and their shape allows them to monitor the fluid collecting apically in the ureter (either as ion or osmosensors, or as stretch receptors) while secreting neurohormones basally into the haemolymph to regulate muscle contractility or ion transport.

Figure 3.3. Mapping domains of gene expression of tubule cell types in the adult.

- a) Lines C324 (shown here) and C374 distinguish subsets of morphologically indistinguishable principal epithelial cells.
 - b) Principal cells of line C324 labelled with FITC secondary antibody against β -galactosidase at higher magnification .
 - c) Lines C710 and C724 (shown here) are expressed uniquely in the stellate cells of adults and larvae. Note the apparent variability of stellate cell shape.
 - d) Confocal image of a single, fluorescently labelled, stellate cell from line C724.
 - e) Line C649 distinguishes the morphologically distinct stellate cells of the initial and transitional segments (HRP-coupled secondary antibody).
 - f) Higher magnification view of the bar-shaped stellate cells.
 - g) Line C325 demonstrates the presence of a small, previously unreported cell type in the lower tubules and ureter. Similar cells are also seen in the midgut.
 - h) Line C710 showing staining of the tiny cells of the lower tubule and ureter. As can be seen, the tiny cells are very much smaller than even the nuclei of the tubule cells. The improved resolution also allows threadlike processes to be viewed on several cells (**Fig 3.4.d**).
-



3.4. Quantification of tubule domains and cell types

Enhancer trapping can provide valuable information on spatial patterning of gene expression, particularly if a sufficiently large panel of lines is screened; however, the expression patterns themselves can additionally be used to deduce the formal genetic subdivision of a tissue, as has recently been described for *D. melanogaster* mushroom bodies (Yang *et al.*, 1995). Furthermore, in this study, it will be shown that the deduced genetic domains in the Malpighian tubules are specified to single cell precision. Additionally, the work provides a methodology for similar work in *Drosophila* and in vertebrates.

Using nuclear counterstaining with ethidium bromide, it proved straightforward to measure distances to expression boundaries by counting principal cell nuclei from either end of the tubule (**Figure 3.4 f**), and to estimate the total number of principal cells within the adult tubule. These gives numbers of principal cells (145 ± 1 and 111 ± 1 in the anterior and posterior tubules, respectively) that are closely comparable with previous studies on larvae and adult (Janning *et al.*, 1986) and embryo (Skaer and Martinez-Arias, 1992). From these data, it was possible to assemble a numerical map of genetic domains defined by gene expression in tubules, and additionally to count the number of principal or stellate cells in each (**Figure 3.4 a-b**). The data are presented in **Table 3.1**, and summarised graphically in **Figure 3.5 a**. It shows that, although the posterior tubules in the adult have been described classically as being longer than the anterior (Wessing and Eichelberg, 1978), this is clearly not the case for the *D. melanogaster* strains we have been using. In this respect, our data agree closely with those of Janning *et al.*, 1986, who found adult anterior tubules both to be longer, and to contain more cells, than the posterior. However we can expand these data, and also show that there is no difference in the length, cell numbers or relative cell types in the main, lower tubule or ureter segments, and that the difference is entirely ascribable to the relative sizes of the initial and transitional segments.

By counting such nuclei, it was possible to reconcile apparently complementary boundaries reported in different lines, and to prove that they were so close together as to be likely to be identical. For example, the number of ethidium-stained principal cell nuclei in the upper domain

of anterior tubules marked by bar-shaped stellate cells (line C649, **Figure 3.3 e**) was 40 ± 0.6 ($n=34$); this corresponded closely with the sum of such nuclei in the initial (22) and transitional (22) segments marked by line C825 (**Table 3.1**); or the region unstained (probably combined initial and transitional segments) in line C568A, containing 44 ± 1.7 ($n=15$) principal cells; or in line c42, containing 44.7 ± 0.8 ($n=35$), as described schematically in **Figure 3.5 b**. The same reconciliation was also possible for posterior tubule. The number of ethidium-stained principal cell nuclei in the upper unstained domain of posterior tubules in line C709, was 6.62 ± 0.17 ($n=53$) principal cells which corresponded closely with the sum of such nuclei in the initial 3.6 ± 0.1 ($n=59$) and transitional 3.4 ± 0.1 ($n=59$) domains marked by line C825 respectively and 6.96 ± 0.13 ($n=53$) altogether (**Table 3.1**). In light of these data, this non-labelled region at the distal end of anterior tubule where the bar-shaped cells are found, turns out to be equivalent to the (combined) initial and transitional segments.

Another example is the number of ethidium-stained principal cell nuclei in the region where no stellate cells were observed in line C724, containing 23 ± 0.6 ($n=26$) principal cells in anterior tubule of adult and 24 ± 0.56 ($n=27$) principal cells in posterior tubules respectively. This corresponded closely with the sum of such nuclei in the lower tubule of anterior tubule 25 ± 0.4 ($n=86$) and posterior tubule 23 ± 0.4 ($n=82$) domains marked by line C507 and C232 (**Figure 3.5 d**); showing no stellate cells were ever observed proximal to main/lower tubule boundary, as the most proximal stellate cell in a tubule can also be seen in **Figure 3.4 e**, supporting these data.

It is also possible to reconcile functional and genetic maps. Ethidium bromide is actively transported by tubule cells: in short-term labelling experiments, it was found that the upper tubule compartment, that does not rapidly stain with ethidium, contains 14 ± 0.4 ($n=25$) secondary cells (as identified by GAL4 line C724 crossed to UASG-GFP). This is in close agreement with line C649, which marks only bar-shaped cells (counterparts of stellate cells in non-transporting initial and transitional segments); containing 13 ± 0.2 ($n=93$) bar-shaped cells. So, from these results, it can be proposed that the region where the bar-shaped cells are located corresponds to the functionally distinct (non-secretory) initial and transitional segments, as presented in **Figure 3.5 c**.

Another example of a reconciliation between genetic and functional maps can be given as follows: in line C507 (which defines the lower tubule domain), there are 118 ± 0.6 ($n=23$) ethidium-stained principal cell nuclei in the unstained portions of adult anterior tubule and 94 ± 0.56 ($n=30$) in posterior tubules. This corresponded closely with the sum of such nuclei in the regions of anterior tubule 108 ± 0.56 ($n=30$) and 85.4 ± 0.56 ($n=18$) posterior tubule that were unstained by alkaline phosphatase histochemistry (**Figure 3.5 d**).

Additionally, it is possible to distinguish nuclei of principal, stellate and “tiny” cells purely on the basis of nuclear size (**Figs 3.4 a-h**), allowing their subsequent study independent of reporter gene marking.

Figure 3.4. Counterstaining with ethidium bromide allows cell numbers to be measured.

a) Reporter gene expression was detected with fluorescein-coupled anti- β -gal secondary antibody (green) in line C724, and counterstained with ethidium bromide. It shows that the relative abundance of principal and stellate cells in the main segment. In addition, the nuclei of the stellate cells are clearly smaller than those of principal cells.

b) Dual staining of the initial segment with line C724 and ethidium bromide shows that it is straightforward to count nuclei unambiguously over the length of the tubule, at least as far as the bifurcation (cf. 3.4d).

c) The sizes of principal and stellate cells' nuclei in the third instar larva of line C724.

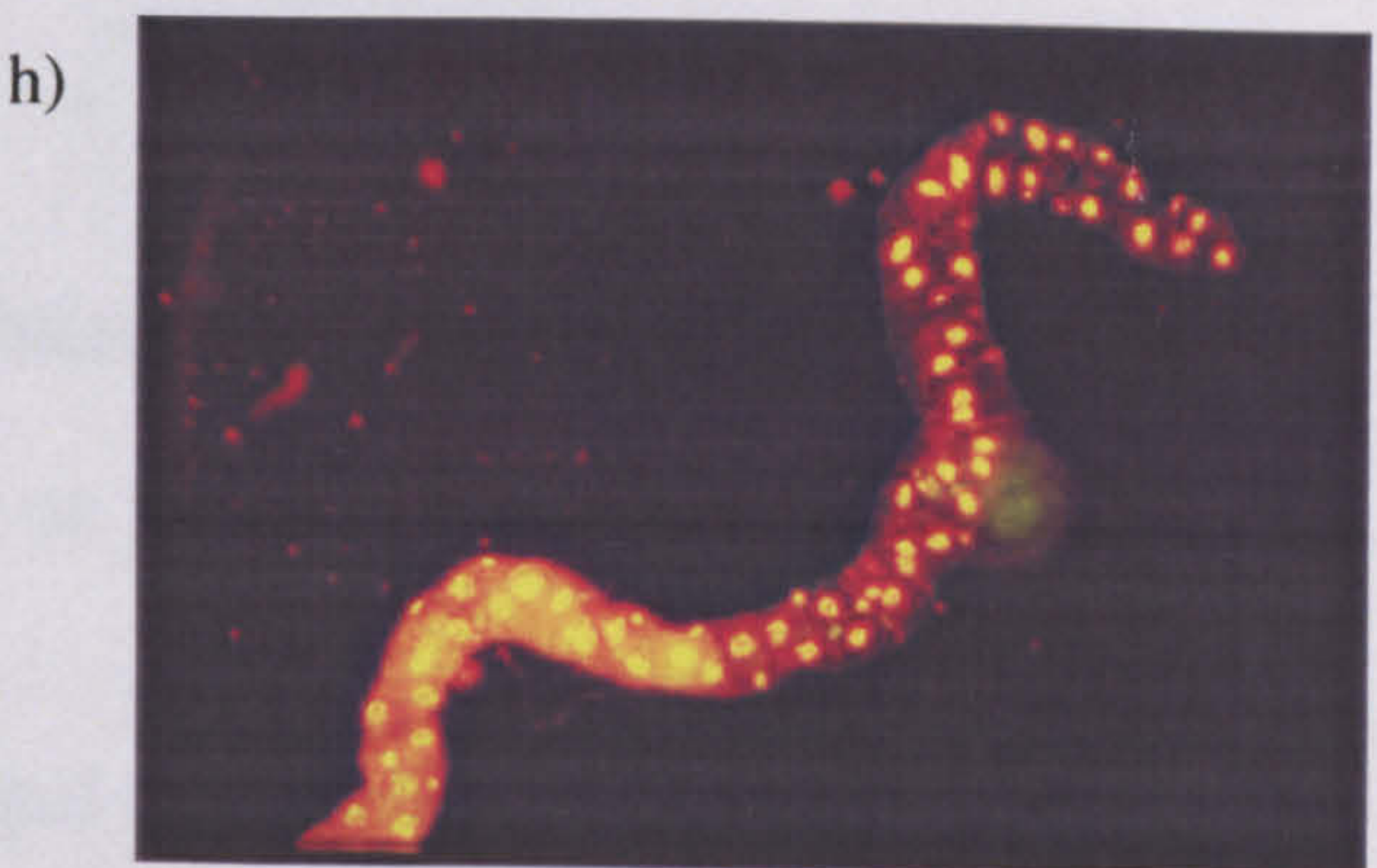
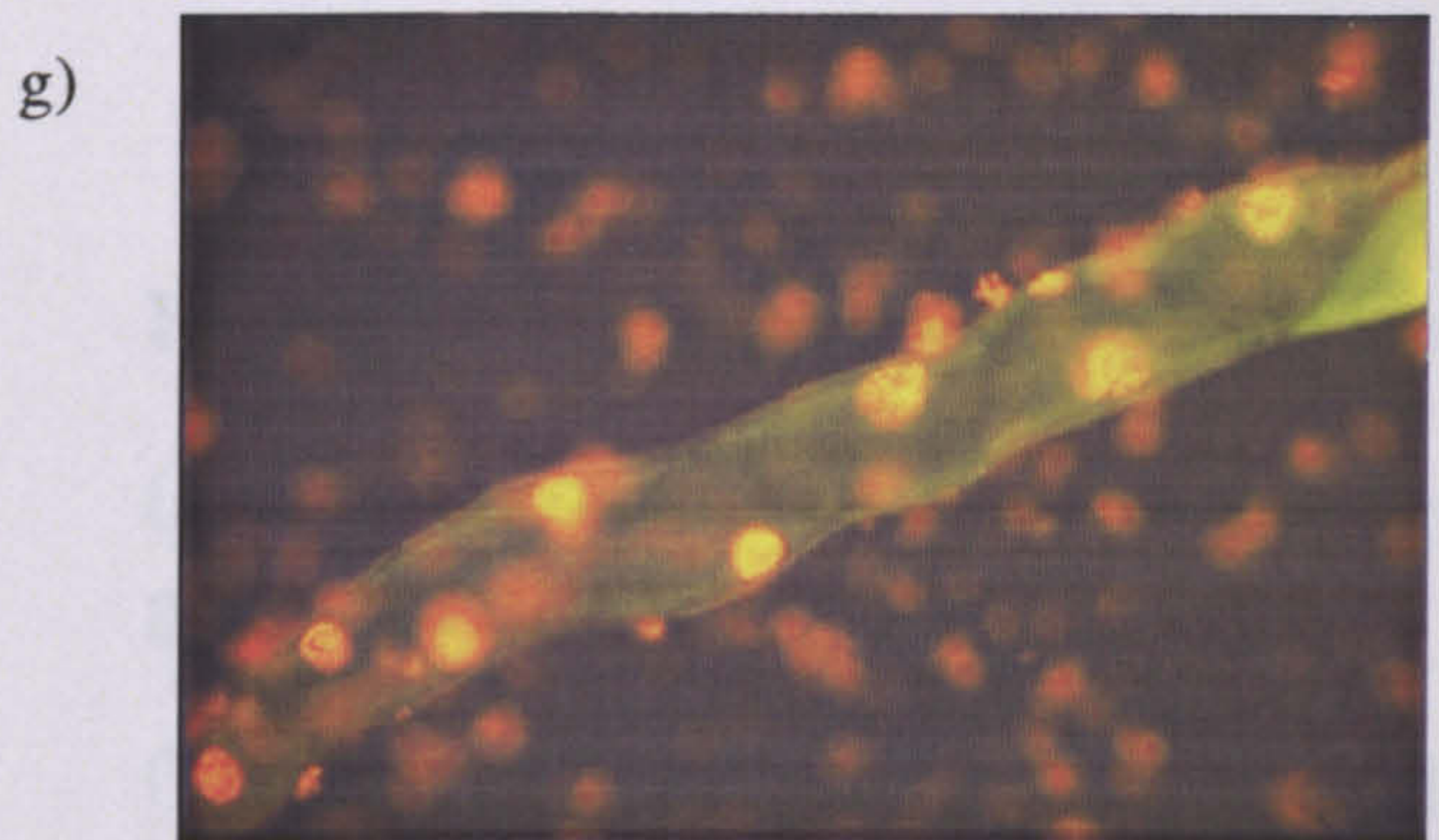
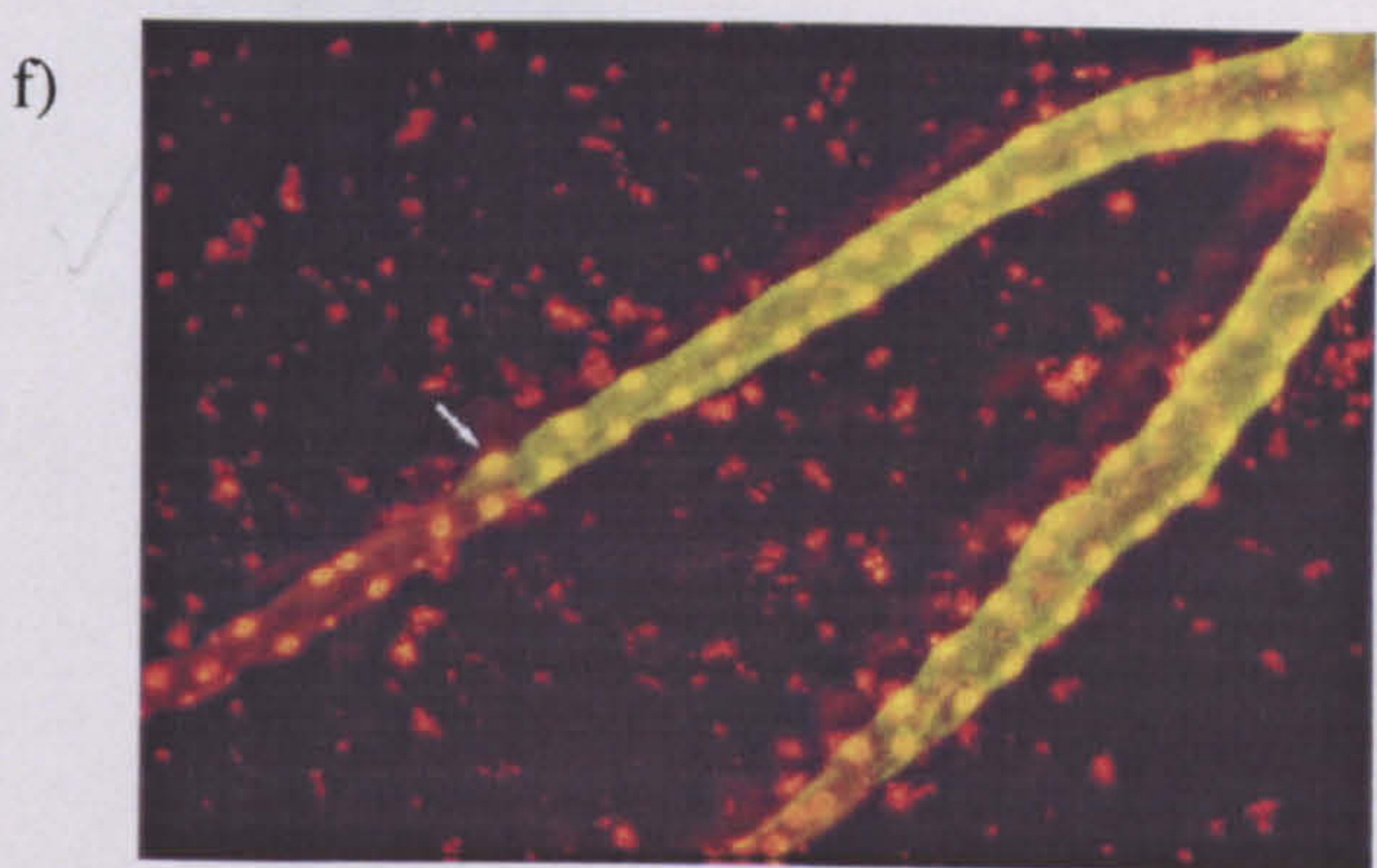
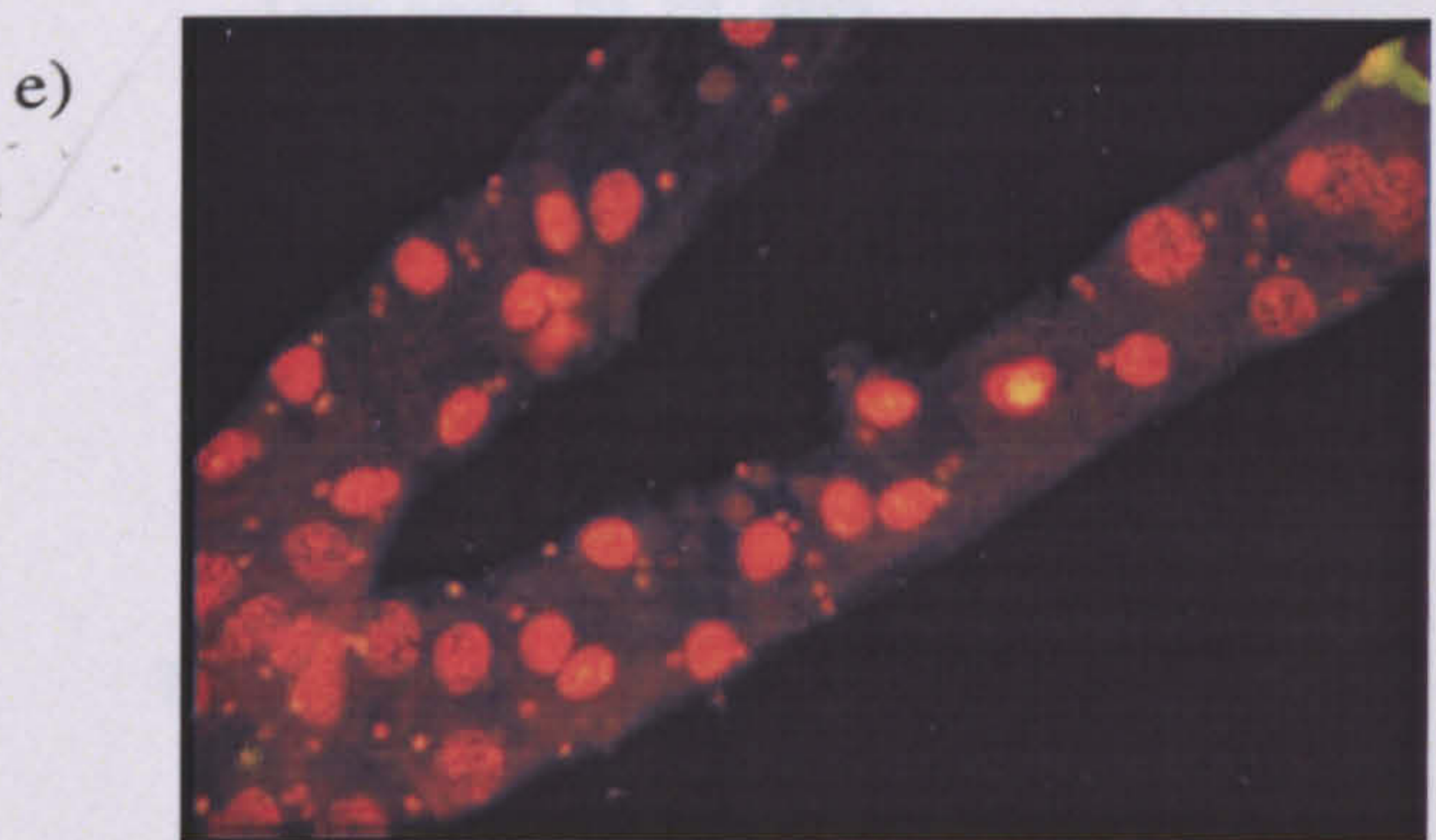
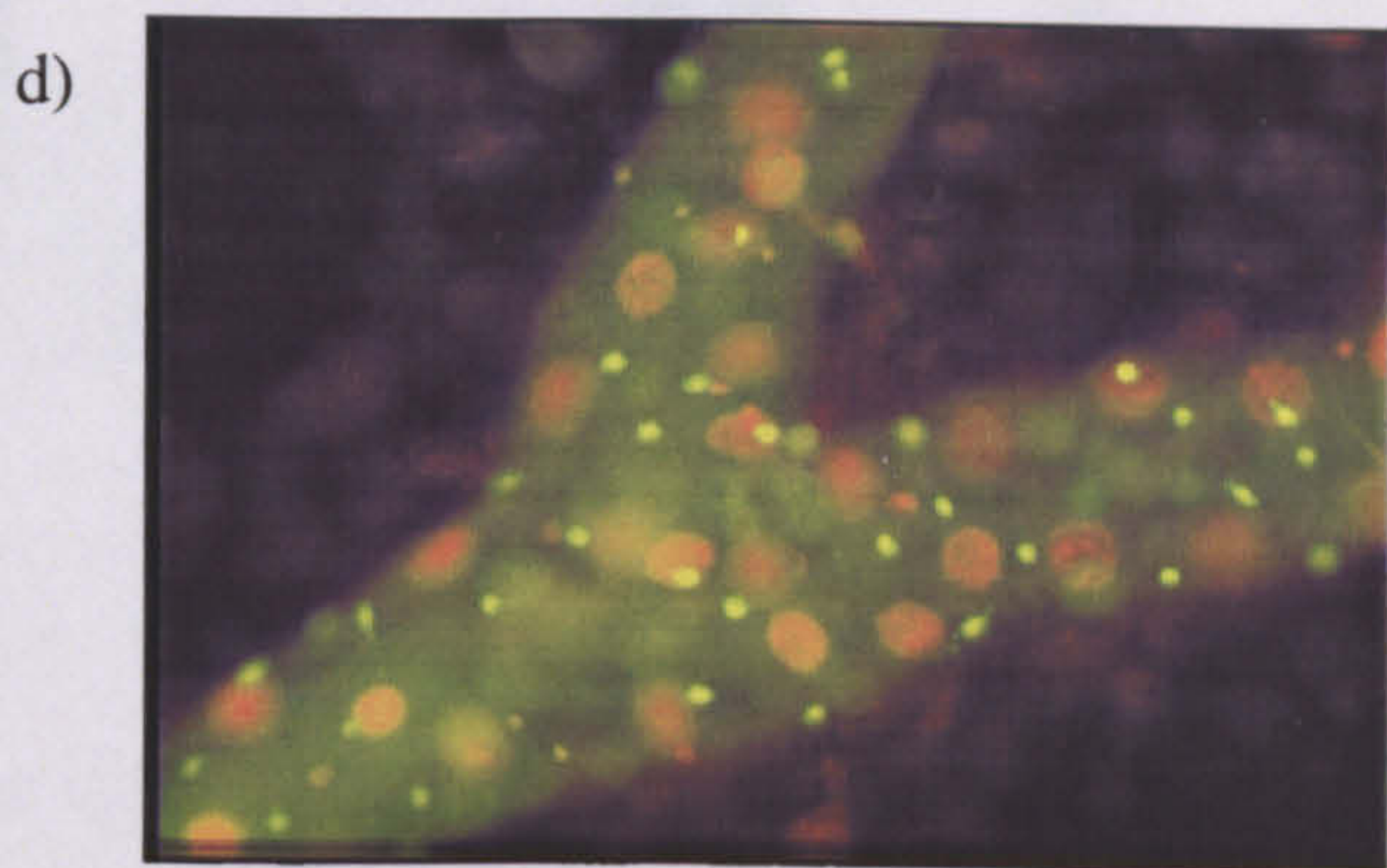
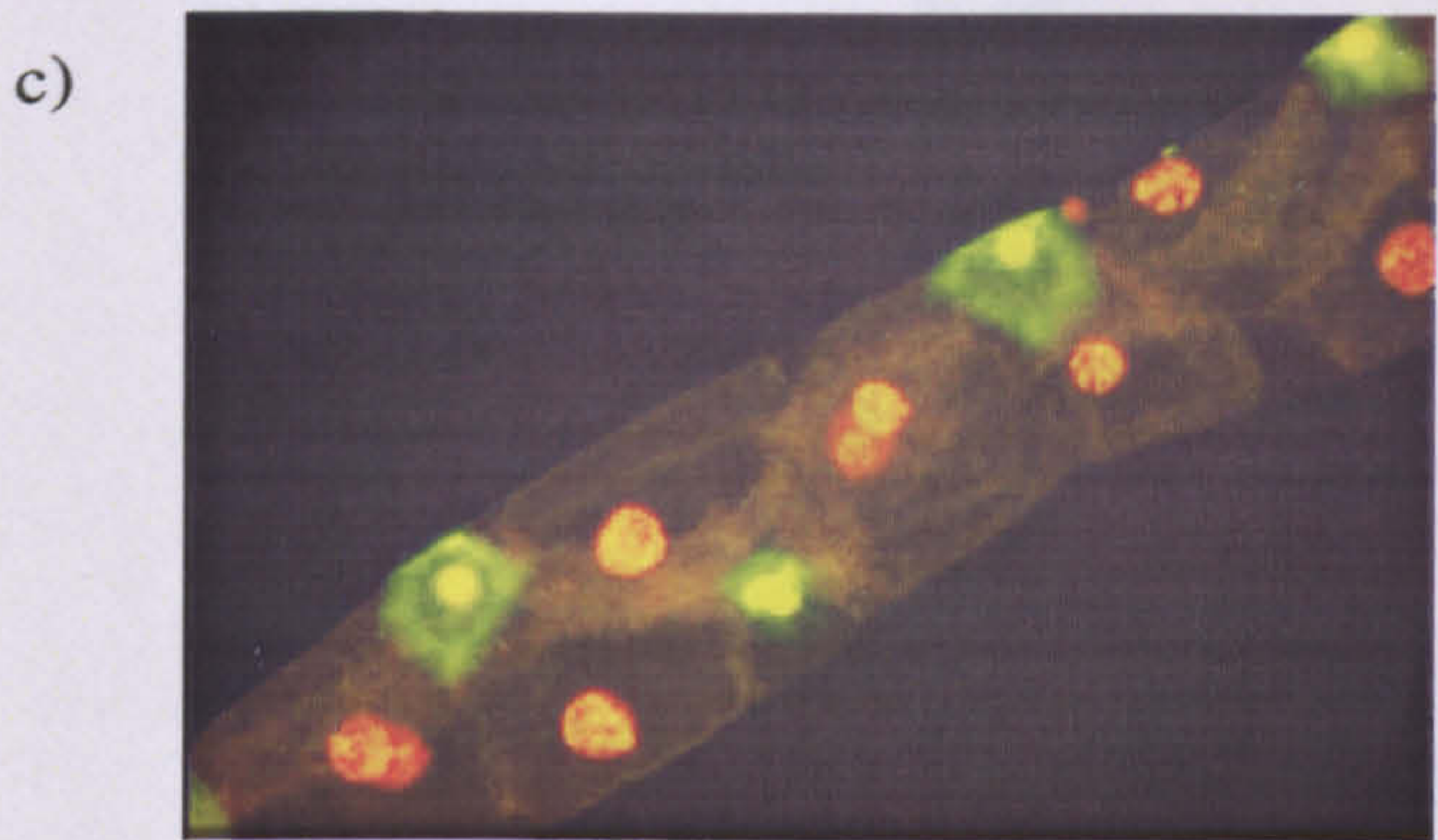
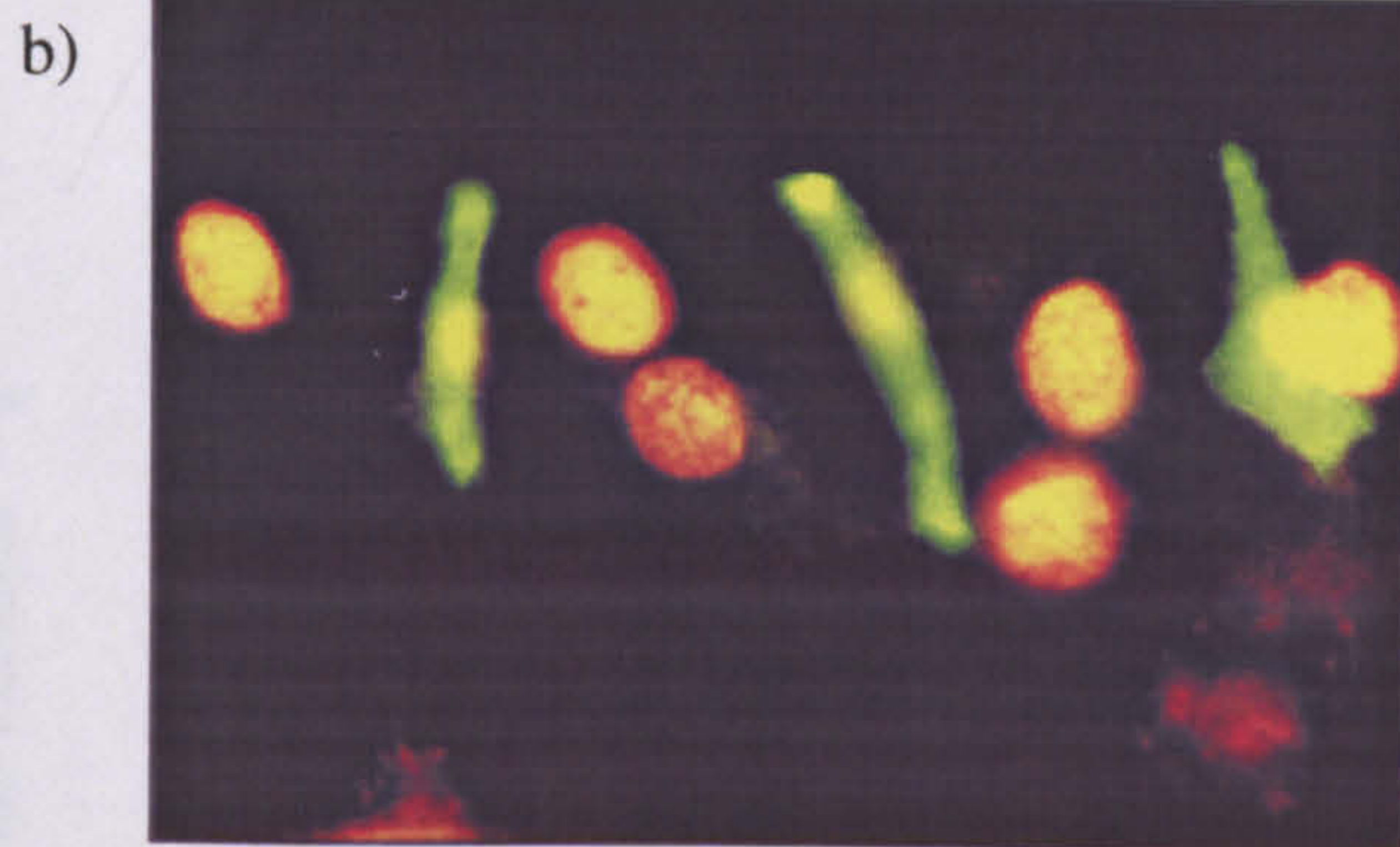
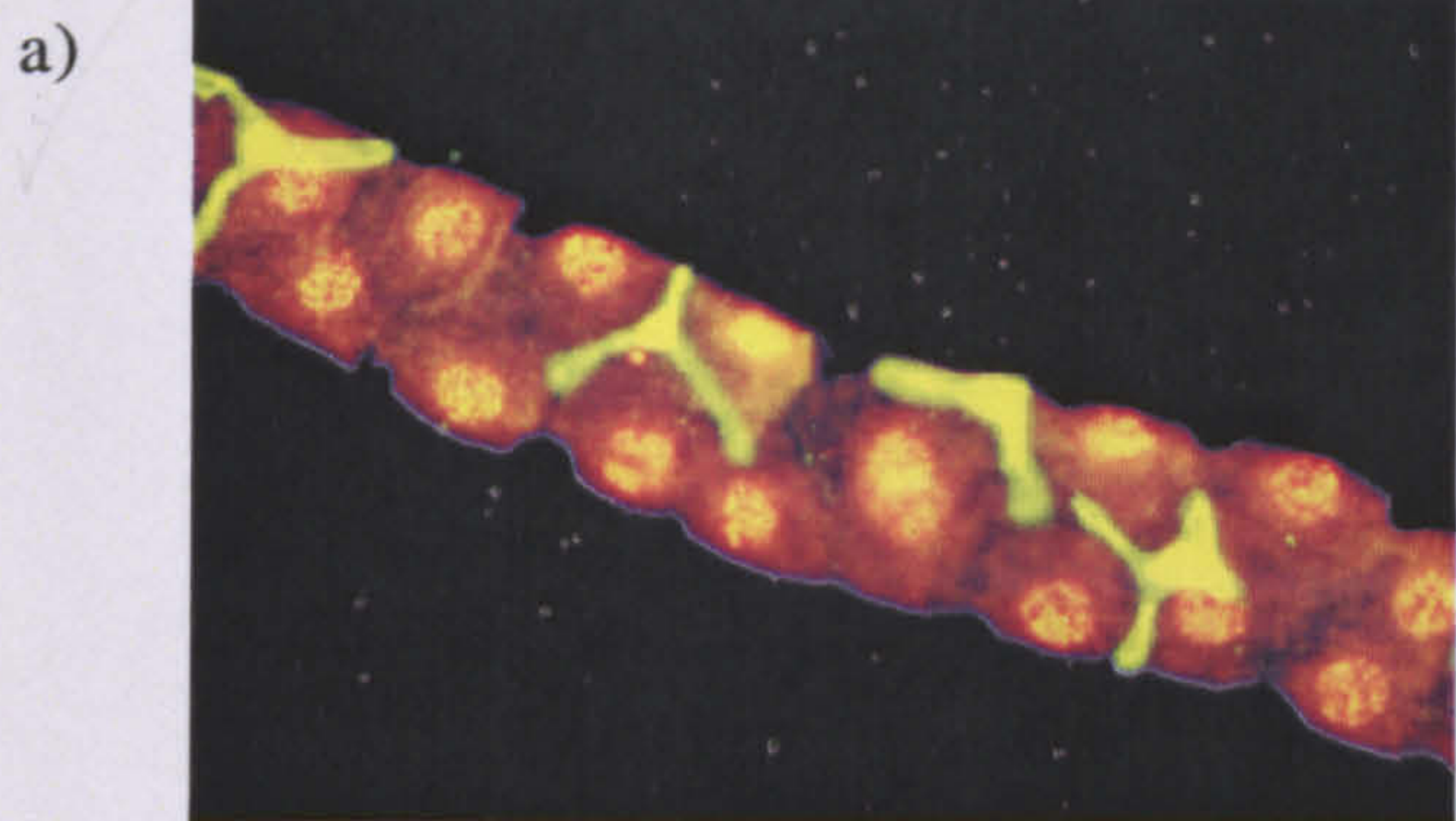
d) In line C710, the tiny cells are very much smaller than even the nuclei of the tubule cells, making them difficult to count.

e) Line 724, showing that stellate cells do not overlap with tiny cells in the lower tubule. A single stained stellate cell is visible at the junction of the lower tubule, and the tiny cell nuclei are visible in the lower tubule.

f) The boundary of β -gal expression, in this case at the main/lower tubule boundary, can be identified to single-cell resolution, and the number of principal cell nuclei to the tubule bifurcation can be counted (line C507).

g) The area at the distal end of posterior tubules (initial and transitional segments) can be determined quantitatively with this method. The tip of the tubule at the left edge of the photograph. The first β -gal positive cell is on the right (line C709).

h) The commonest staining pattern of tubules is of main segment, but not initial or transitional segments (line C303)



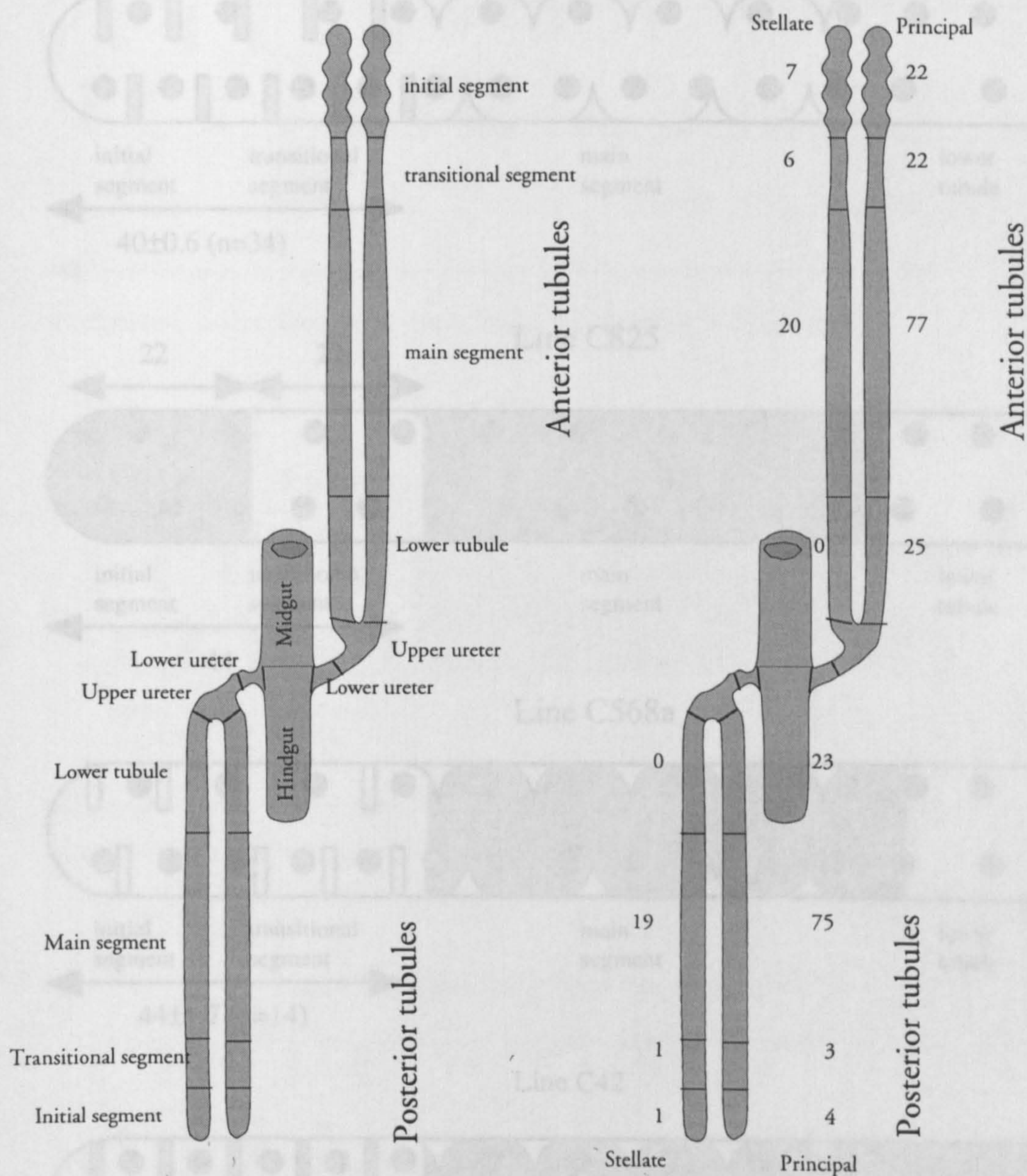


Figure 3.5 a. Summary of regional architecture of adult tubule.

(a) Subregions that can be distinguished on the basis of enhancer-driven gene expression.

(b) Size of subregions, counted as principal cell nuclei (on the right) and stellate cell numbers for each subregion.

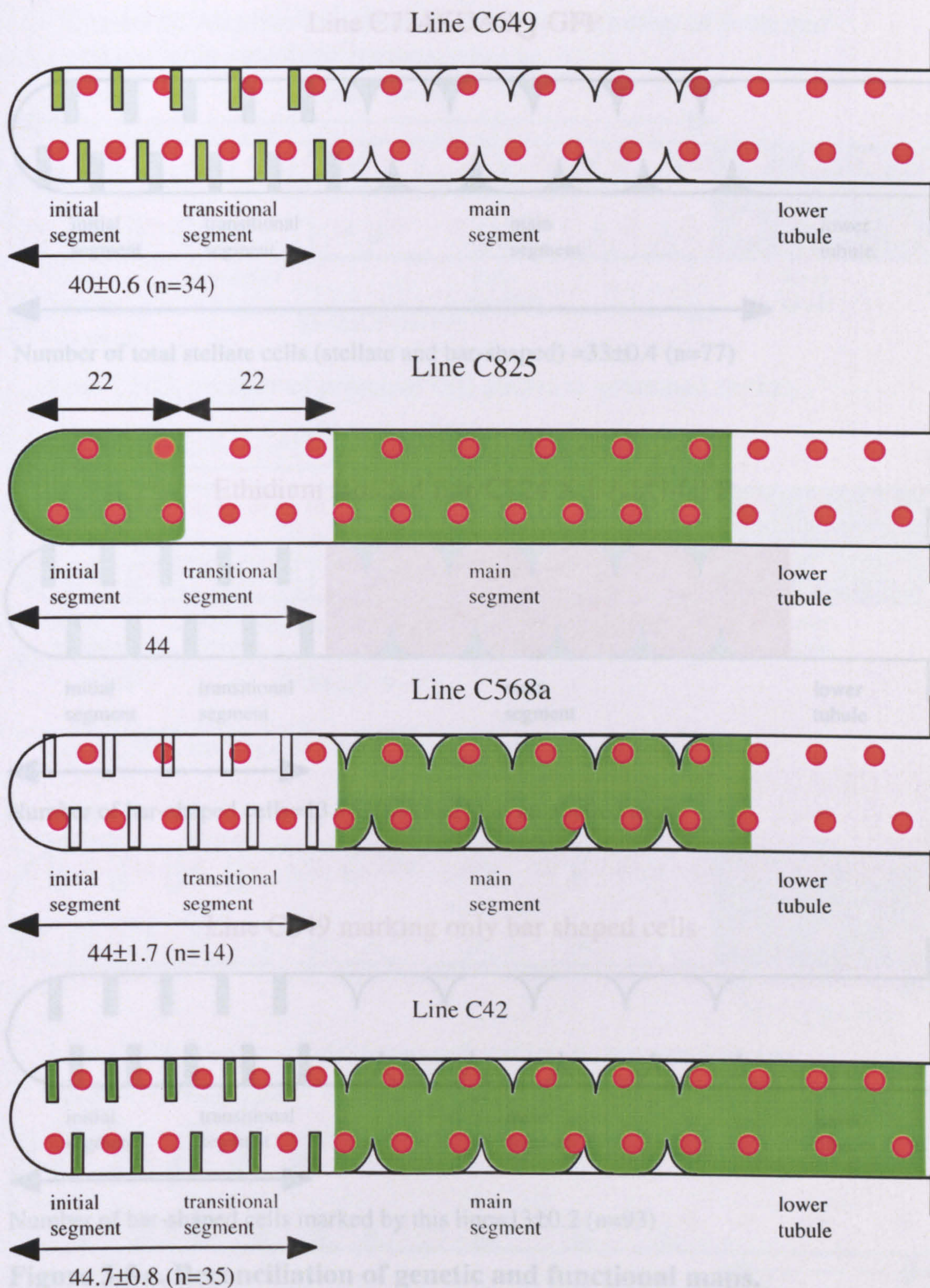


Figure 3.5 b. Reconciliation of complementary boundaries reported in different lines.

The number of ethidium-stained principal cell nuclei in the upper region of anterior tubule marked by bar-shaped cells (line C649) corresponds closely with the sum of such nuclei in the initial and transitional compartments marked by line C825; or the region unstained in line C568a or in line C42. Orange circles denote principal cell nuclei; green denotes GAL4 expression pattern.

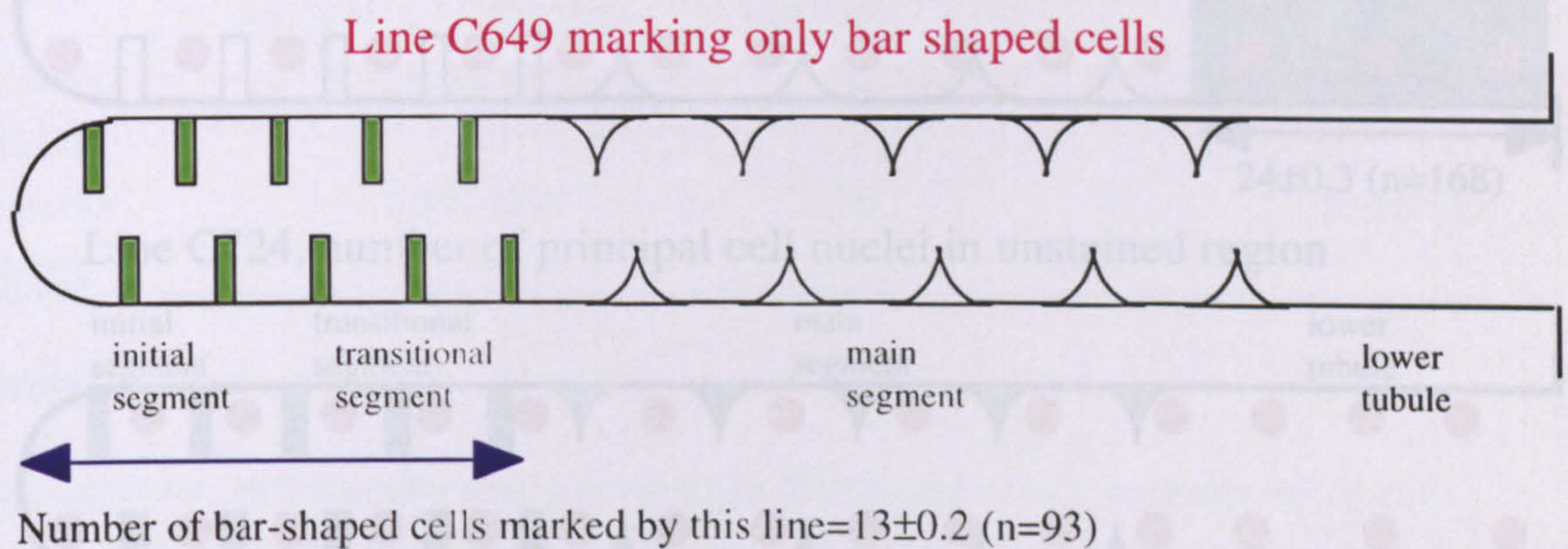
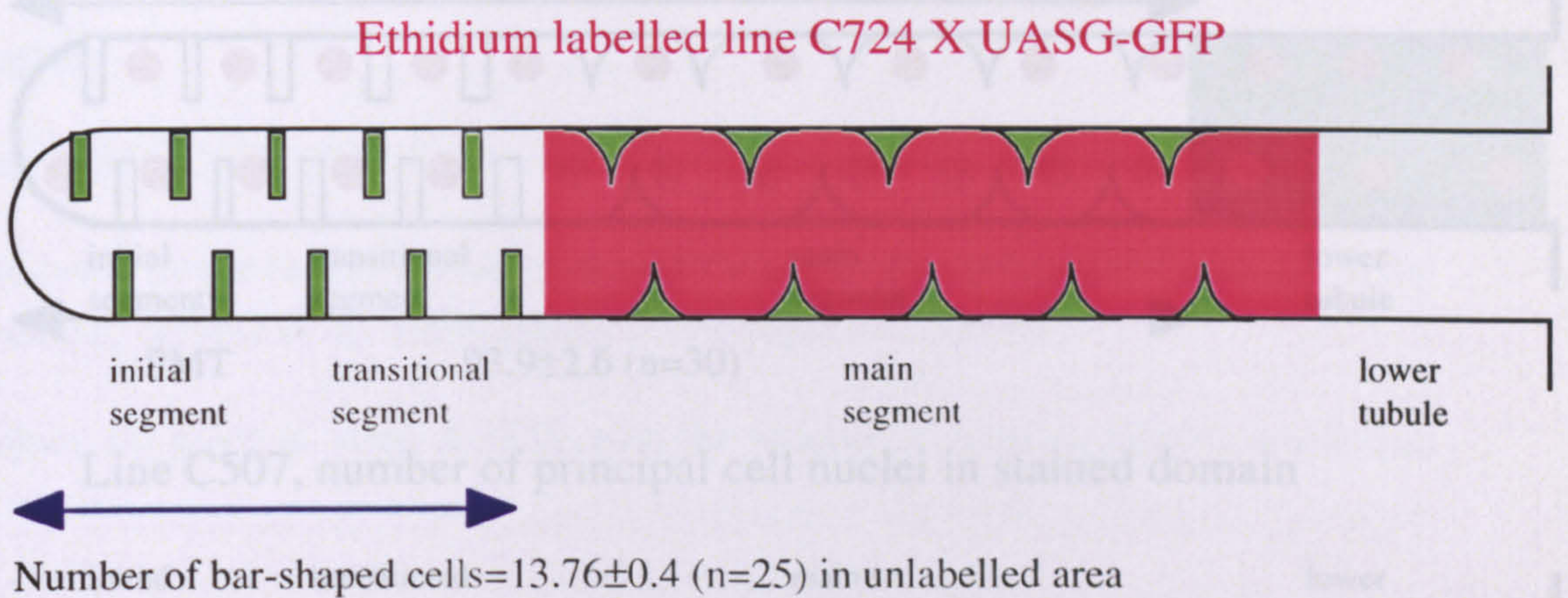
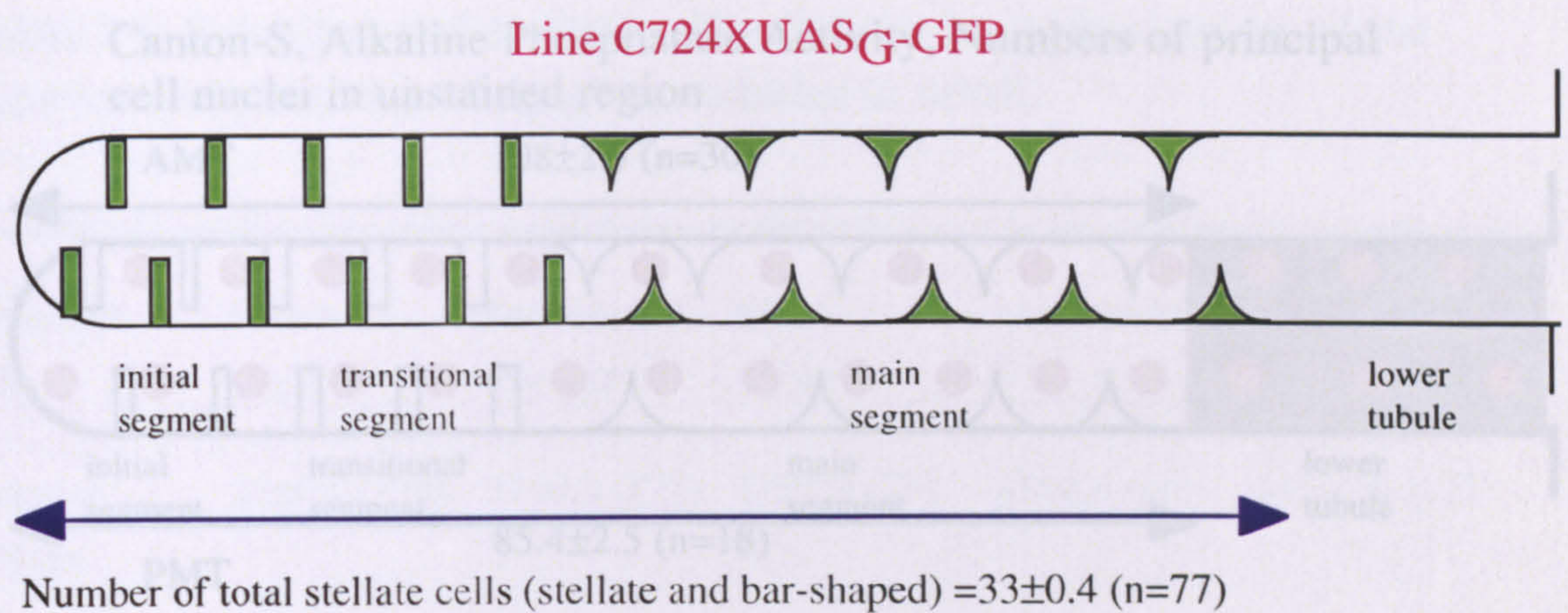
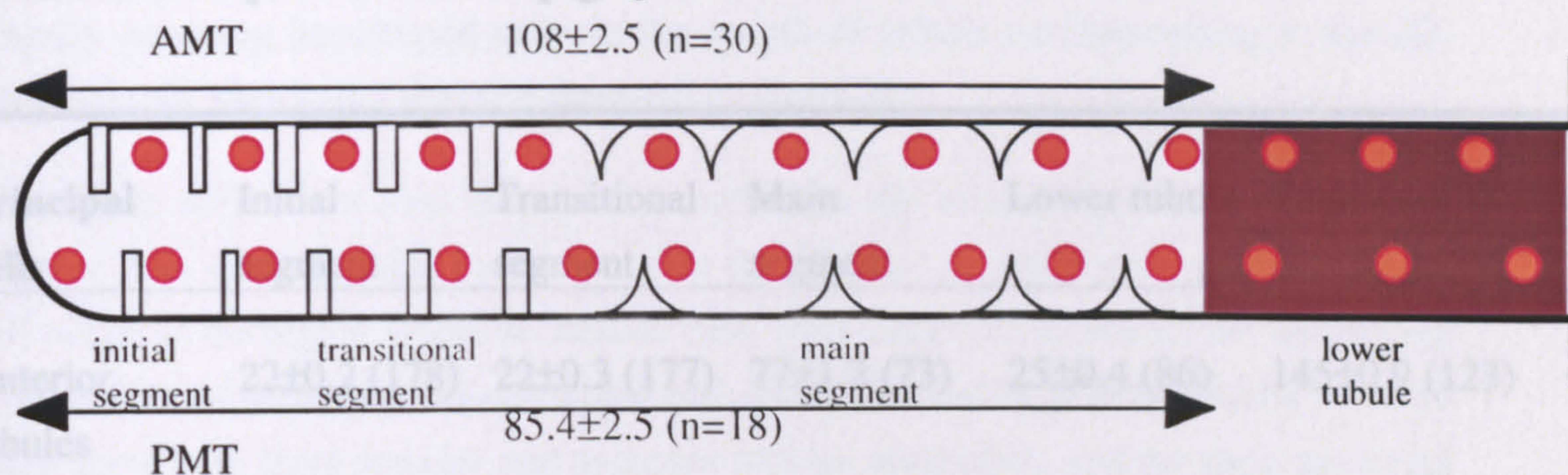


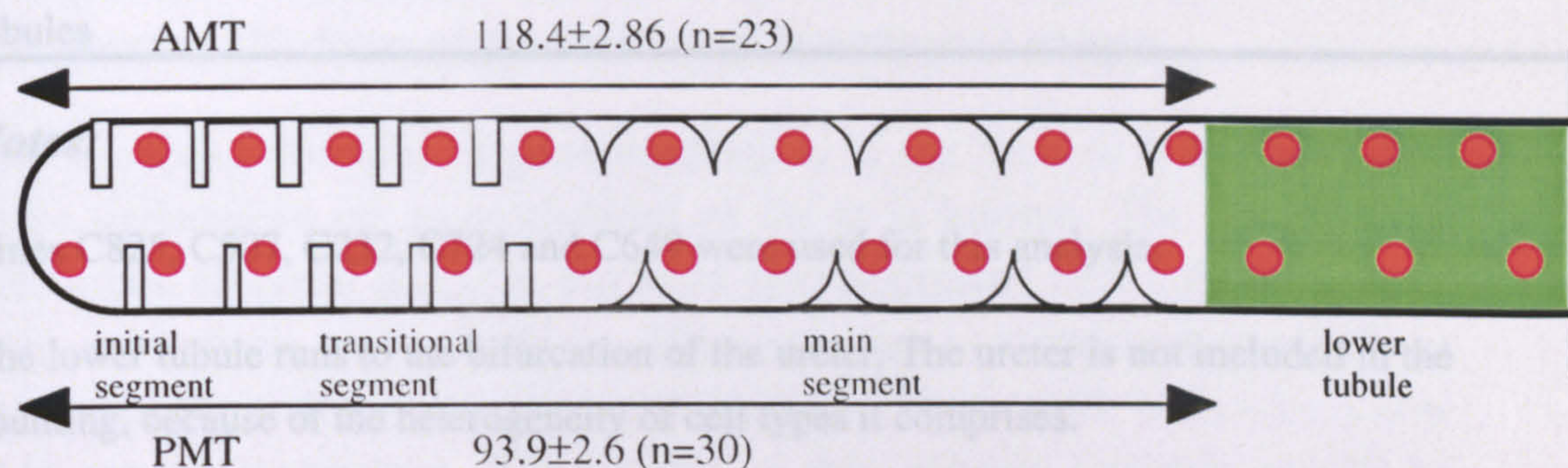
Figure 3.5 c. Reconciliation of genetic and functional maps.

Ethidium bromide uptake in living tubules marks by exclusion a region indistinguishable from the (initial+transitional) domain identified by line C649. The difference in the number of type II cells excluded from the ethidium transporting compartment (middle diagram) does not differ significantly from the number of bar-shaped cells in line C649 (bottom panel) that marks the initial and transitional segments (see **figure 3.5b**) (Students' t-test, two tailed, taking a critical level for $P=0.05$).

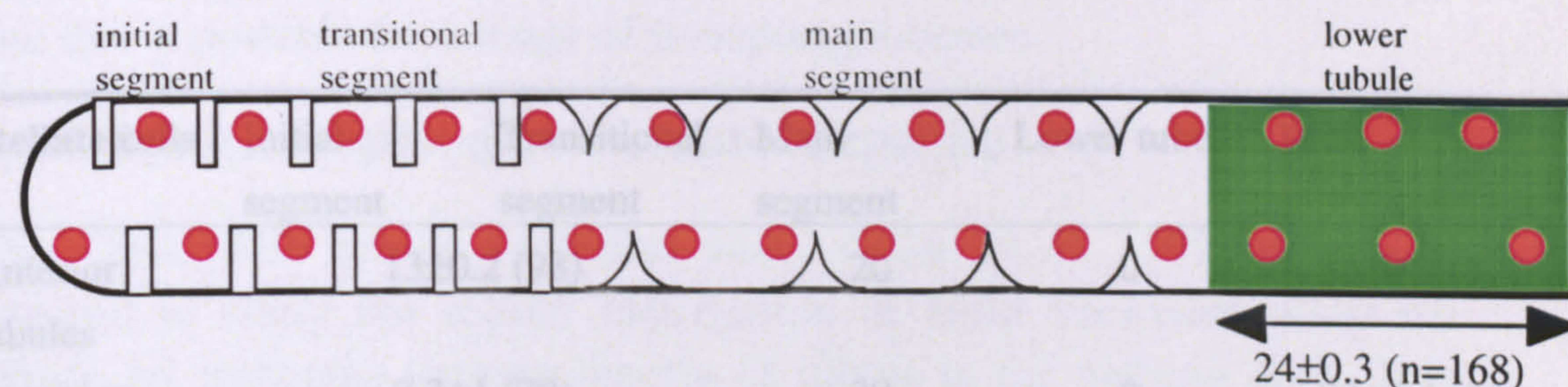
Canton-S, Alkaline Phosphatase Activity, Numbers of principal cell nuclei in unstained region



Line C507, number of principal cell nuclei in unstained region



Line C507, number of principal cell nuclei in stained domain



Line C724, number of principal cell nuclei in unstained region

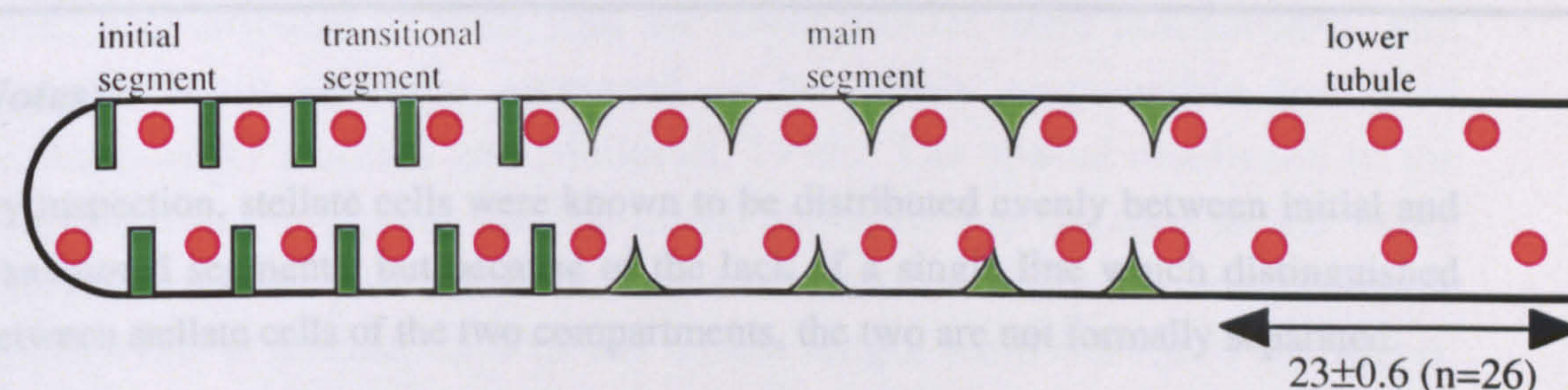


Figure 3.5 d Reconciliation of genetic and functional maps.

In adults, alkaline phosphatase activity maps closely to a domain identical to that labelled by line C507 or that containing no stellate cells, was observed in line C724. Brown denotes alkaline phosphatase activity, green denotes GAL4 expression pattern. Abbreviations: AMT: Anterior Malpighian tubule; PMT: Posterior Malpighian tubule

Table 3.1. Principal and stellate cell numbers in each segment of the anterior and posterior Malpighian tubules in adult.

Principal cells	Initial segment	Transitional segment	Main segment	Lower tubule	Total
Anterior tubules	22±0.2 (178)	22±0.3 (177)	77±1.2 (73)	25±0.4 (86)	145±0.9 (123)
Posterior tubules	3.6±0.1 (59)	3.4±0.1 (59)	75±2.4 (13)	23±0.4 (82)	111±1.0 (120)

Notes:

Lines C825, C507, C232, C724 and C649 were used for this analysis.

The lower tubule runs to the bifurcation of the ureter. The ureter is not included in the counting, because of the heterogeneity of cell types it comprises.

Values are given as mean ± SEM, with the number of separate tubules counted in brackets.

Stellate cells	Initial segment	Transitional segment	Main segment	Lower tubule	Total
Anterior tubules	13±0.2 (93)		20	0	33±0.4 (77)
Posterior tubules	2.3±1 (79)		20	0	22±0.4 (59)

Notes:

By inspection, stellate cells were known to be distributed evenly between initial and transitional segments, but because of the lack of a single line which distinguished between stellate cells of the two compartments, the two are not formally separated.

Numbers of stellate cells in the main segment were calculated by subtraction of cell numbers from line C649 (which specifically marked stellate cells of initial and transitional segments), from totals obtained from lines C710 or C724 (which marked all stellate cells). However, bar-shaped cells are not subdivided formally in this table

due to the lack of a distinguishing marker line. The distribution of bar-shaped cells as 7 (in initial segment) and 6 (transitional segment), shown in Figure 3.5 a, is based on visually counting bar-shaped cells in the length of tubule corresponding to the 22-principal cell long initial segment reported by line C825.

No stellate cells were ever observed proximal to the main/lower tubule boundary (Figure 3.4e cf. 3.4.a) due to the fact that the number of ethidium-stained principal cell nuclei in the region where no stellate cells were observed in line C724, containing 23 ± 0.6 (n=26) principal cells in adult and 24 ± 0.56 (n=27) principal cells in third instar larva both from anterior and posterior tubules altogether, and the most proximal stellate cell in a tubule can be seen in Figure 3.4e.

3.5. Reconciliation of genetic and functional maps

This mapping domains of gene expression in the tubules is the first such to be undertaken on an epithelium as far as is known. The results have confirmed classical analysis, but have also suggested new subdivisions of both subregions and cell types. This provides —at present uniquely in epithelial physiology— a rigorous coordinate framework with which new data can be reconciled. The weight of this genetic analysis would be increased further if physiological properties could be mapped to the same genetic compartments identified by enhancer trap lines. Here, it is shown that this is possible for a range of transport processes.

3.5.1. Spatial mapping of transport capacity

The *Drosophila* tubule secretion assay (Dow *et al.*, 1994b) can be adapted to study the spatial distribution of fluid transport along the tubule, by including varying lengths of tubule in the bathing drop. This revealed that the initial segments of the anterior tubules did not secrete detectable amounts of fluid, that the lower tubules were reabsorptive, and that the main segment appeared to be solely responsible for fluid production (O'Donnell and Maddrell, 1995). The spatial resolution of the assay is relatively poor, but the initial segment was defined as the white terminal region, and the boundary between secretory and reabsorptive regions fell at around 65% of the tubule length from the tip to the ureter. This would correspond closely with the main/lower tubule distinction described earlier (Figs. 3.2 h & 3.4 f).

In related work, a putative aquaporin from tubules has been cloned, and an antibody against vertebrate basolateral intrinsic protein (a MIP

identified in skeletal muscle sarcolemma, astrocyte feet and kidney collecting duct principal cell basolateral membrane: (Verbavatz *et al.*, 1994) shown to specifically label stellate cell basolateral membranes (Dow *et al.*, 1995). Given that this work shows that stellate cells are found only in the secretory, and not the reabsorptive, tubule regions, it is thus tempting to speculate that stellate cells play an essential role in fluid secretion.

3.5.2. Alkaline phosphatase activity in the tubules

The genomic DNA flanking several of these insertions has been plasmid-rescued and sequenced. In the case of lines C507 and C232, the neighbouring transcription unit appears to encode a form of alkaline phosphatase (Yang *et al.*, in preparation). Accordingly, tubules were fixed and stained for alkaline phosphatase, using the colour development reagents of the Boehringer Mannheim DIG labelling and detection kit according to the manufacturer's instructions (DIG is normally detected with an alkaline phosphatase-coupled antibody). The staining pattern (**Figure 3.6.a**) clearly matches closely that reported by the P{GAL4} insertion in the gene (**Figures 3.4f** and **3.2h**).

3.5.3. Organic solute transport

One of the functional properties ascribed to insect tubules is the excretion of organic metabolites, and several dyes are actively transported by tubules (Maddrell *et al.*, 1974). The cationic dye rhodamine 123 was observed to be excreted by tubule principal cells, then secondarily reabsorbed from the lumen into the stellate cells, implying distinct roles for these cells in solute transport (Meulemans and De Loof, 1992), and supporting their separate patterns of gene expression reported here (**Figs 3.2, 3.3, 3.4, 3.6i. & 3.6j**). When reproducing these effects, it was observed that immediately after adding rhodamine 123 to the tubules, the dye appeared in the lumen of the main segment only, suggesting that the initial, transitional and lower tubule segments (**Figure 3.6b-d**) play no part in this organic solute transport. The initial/transitional segments mapped by exclusion in this way was much bigger in anterior than posterior tubules (**Figs 3.6.c** cf. **3.6.b**), confirming the homology between anterior and posterior tubule initial and transitional segments. Again, the boundaries reported in this functional assay match those obtained from analysis of gene expression as closely as can be resolved (cf. **Figs 3.2a-b**

& 3.2d-h). The same experiment was repeated with ethidium bromide on the anterior tubules labelled with GFP only in stellate cells. It confirmed that the compartment marked by bar-shaped cells is quantitatively identical to that mapped by ethidium bromide transport (**Figure 3.5 c**).

3.5.4. V-ATPase distribution

V-ATPases are ubiquitous to higher organisms, where they acidify the intracellular endosomal compartments. However, V-ATPase has also been implicated as performing vectorial work on the apical plasma membranes of several epithelia, such as kidney tubule (Gluck and Nelson, 1992), frog skin (Harvey, 1992), and many insect epithelia including Malpighian tubules (Klein, 1992). In separate work, genes for five V-ATPase subunits have been cloned and sequenced, and lethal alleles for three of them have been identified. The *vha55* gene, encoding the 55 kDa B-subunit of the V-ATPase, has been inactivated by a P-element insertion into a large intron at the 5' end of the ORF, producing a lethal recessive phenotype (Davies *et al.*, 1996). The P-element carries a *lacZ* reporter gene, in this case coupled to a nuclear targeting signal, that allows the areas of high V-ATPase expression in the insect to be mapped, showing that the reporter gene is expressed at high levels in those epithelia in which plasma membrane V-ATPases have been implicated (Dow, 1994). Here, the data pertaining to the tubules show that *LacZ* expression in the initial and transitional segment (**Figure 3.6e**) and the ureter is much weaker than in the main segment. Furthermore, the nuclei labelled within the tubules (**Figure 3.6g**) are entirely the larger, principal cell nuclei; stellate cell nuclei do not stain (cf. **Fig 3.4g**). This provides not only clear supporting evidence for the initial/main/lower tubule division, but also suggests a clear difference in function between principal and secondary cells in Malpighian tubules, implying that active cation transport is confined to principal cells (Davies *et al.*, 1996).

Additionally, even the weaker staining of the ureter shows a clear subdivision between proximal and distal ureter (**Figure 3.6.f**) which respects the enhancer trap boundary shown in **Figs 3.2g** and **3.2.h**.

3.5.5. HRP immunoreactivity

The characteristic small size and shape of the tiny cells suggested a number of possible roles, including a possible neuroendocrine role.

Antisera against horseradish peroxidase are known to label selectively central and peripheral neurons of several insect species, including *Drosophila* (Jan and Jan, 1982; Siddiqui and Culotti, 1984), because they fortuitously recognise a cell-surface carbohydrate moiety (Kurosaka *et al.*, 1991). If the tiny cells were of neuronal origin, therefore, it was very likely that they would be labelled by antibodies to HRP. As can be seen, this is the case (**Figure 3.6h**). Although this falls short of a rigorous proof of a neuroendocrine role for these cells, it clearly validates the tiny cells reported in lines C325, C374 and C710 (**Figures 3.3.g., 3.3.h. and 3.4.d.**).

Figure 3.6. Functional mapping of tubule regions.

a) Alkaline phosphatase staining of lower tubule. This shows a very close overlap with line C507 (cf. **Figure 3.2.h**); the flanking gene is homologous with vertebrate alkaline phosphatases (Yang *et al.*, in preparation).

b,c,d) Short-term labelling with rhodamine 123 identifies the main segment as the region responsible for transport of this organic molecule. b) posterior tubule, c) anterior tubule, d) lower tubule.

e) Initial and transitional segments express V-ATPase at a far lower level than the main segment, as detected by a reporter gene inserted into the *vha55* gene in the P[LacW] lethal line *vha55j2e5* (Davies *et al.*, 1995). This corresponds to the boundary reported by lines C748 and C303 in **Figs 3.1 i-j** (and 155Y, C709 and C776 in **Figs 3.2 a-b**).

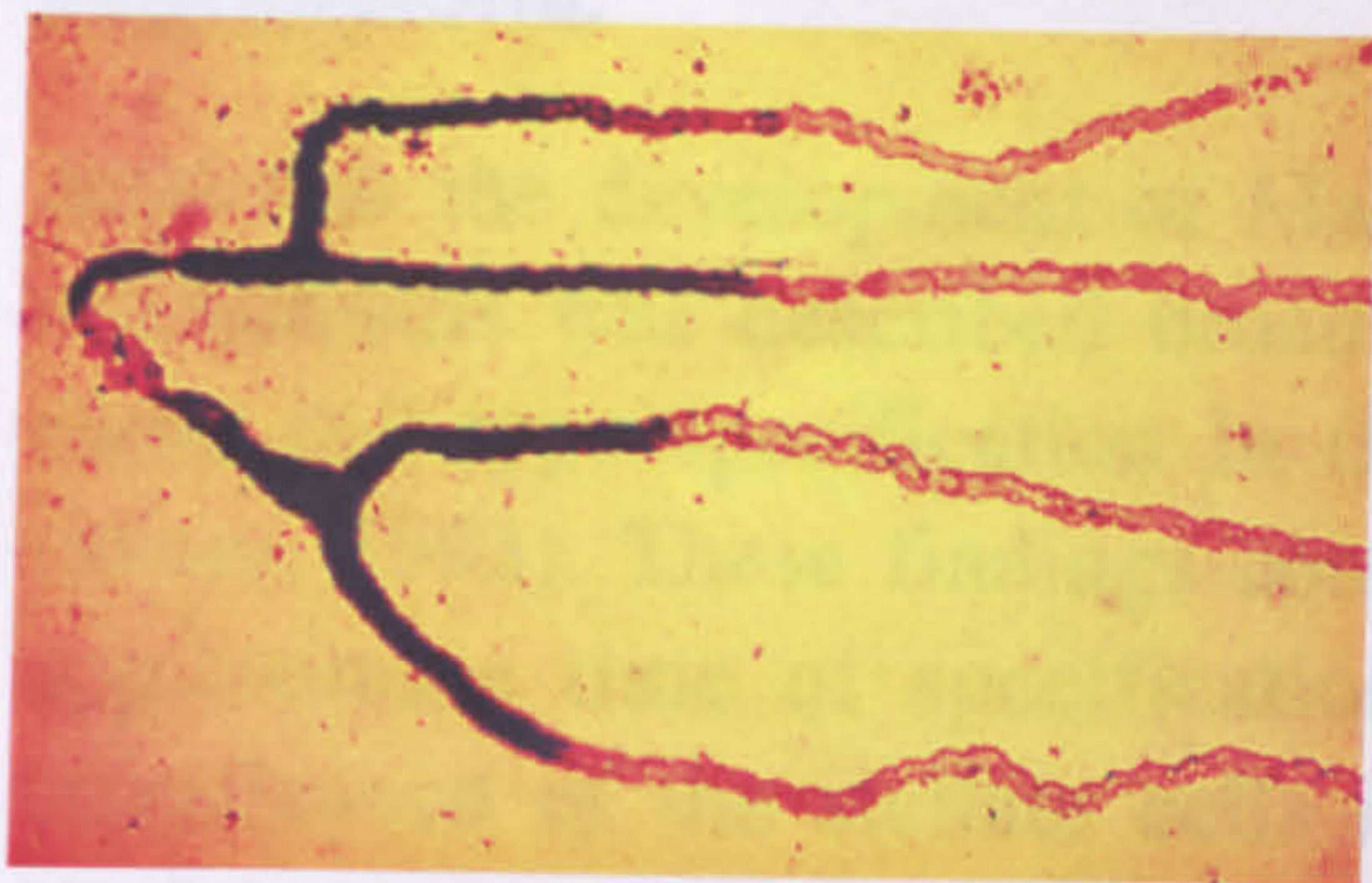
f) A boundary midway along the ureter is marked by a change in the size of nuclei labelled by the P{LacW} lethal line *vha55j2e5*. This corresponds to the boundary reported by lines C649 and C601 in **Figs 3.2 i and 3.2 j**.

g) Within the main segment, only principal cell nuclei are labelled in P{LacW} lethal line *vha55j2e5*. Smaller secondary cell nuclei do not stain. This corresponds to the distinction between principal and stellate cells implied in **Figs 3.3 a-b and 3.3 c-d**.

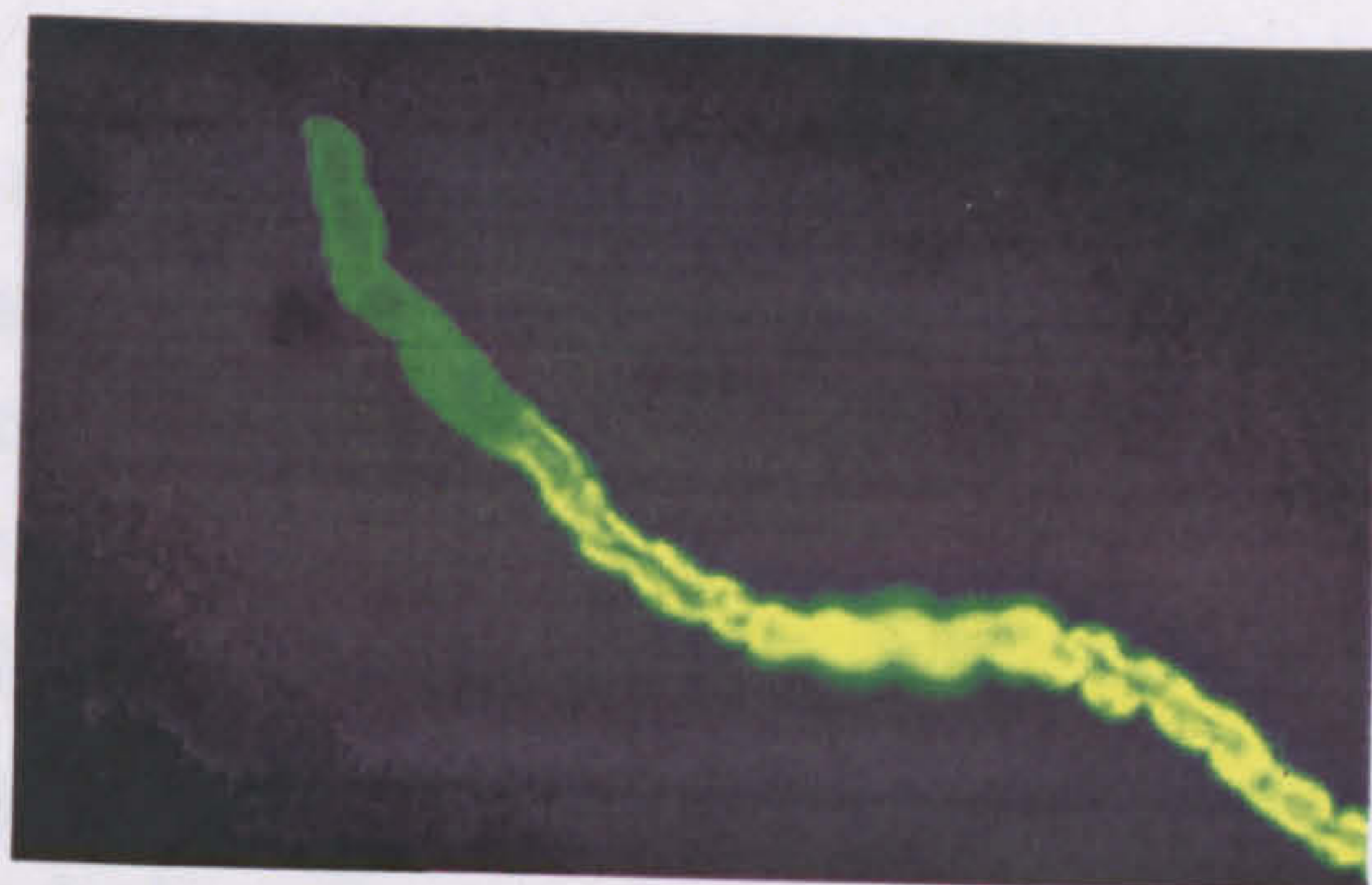
h) The tiny cells are labelled by antibodies to horseradish peroxidase, which recognise an epitope on a *Drosophila* nervous-system-specific protein (in fact, a neuronal-specific β -subunit of the Na⁺,K⁺ ATPase). This corresponds to the tiny cells identified by line C325 in **Figure 3.3.g** and C710 in **Figures 3.3 h and 3.4 d**.

i,j) Longer-term labelling with rhodamine 123 shows concentration of label first in principal cells after 1 hour (i), then in stellate cells after 2 hours (j), showing that they are functionally distinct, as suggested previously (Meulemans and de Loof, 1992).

a)



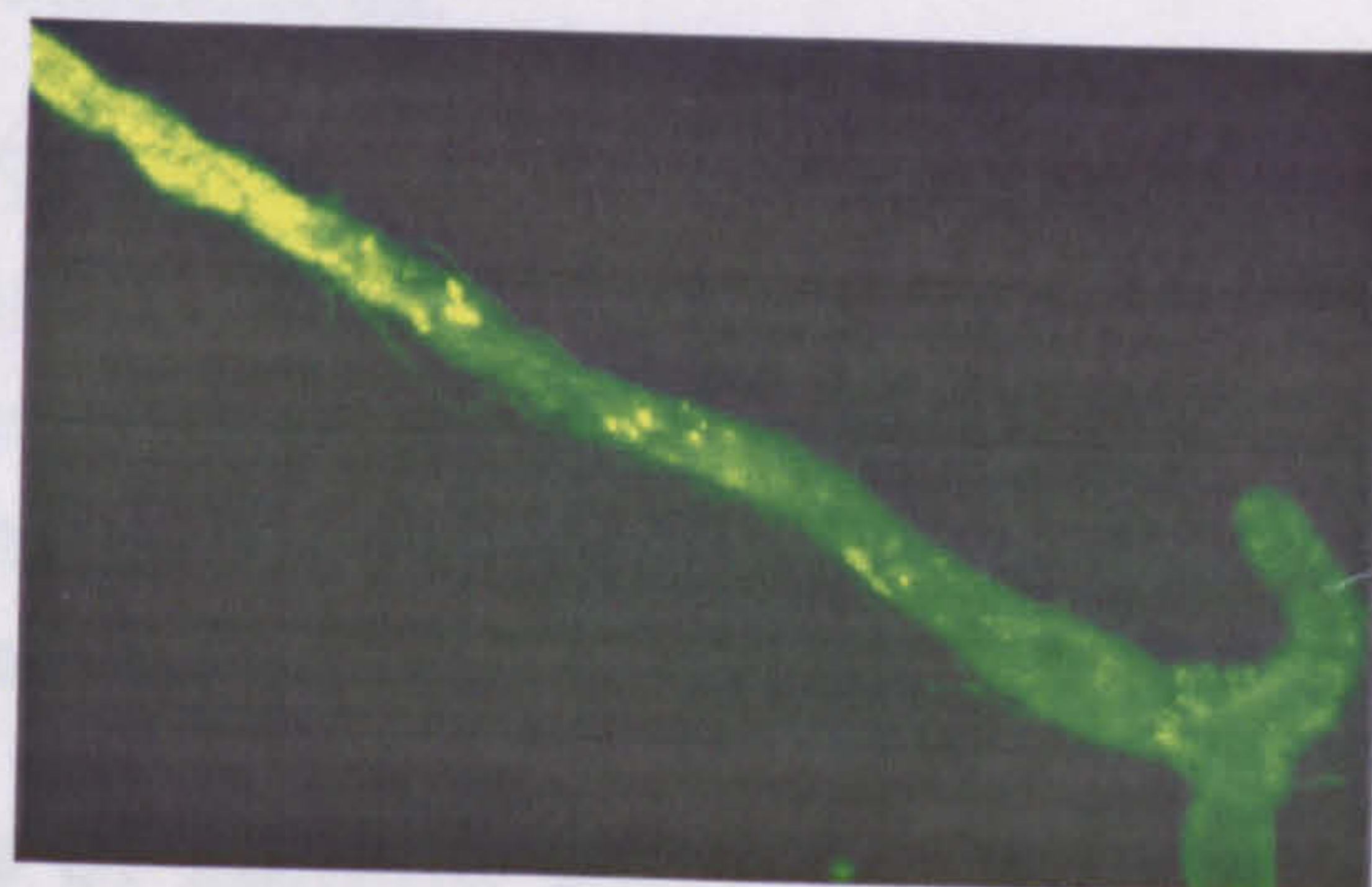
b)



c)



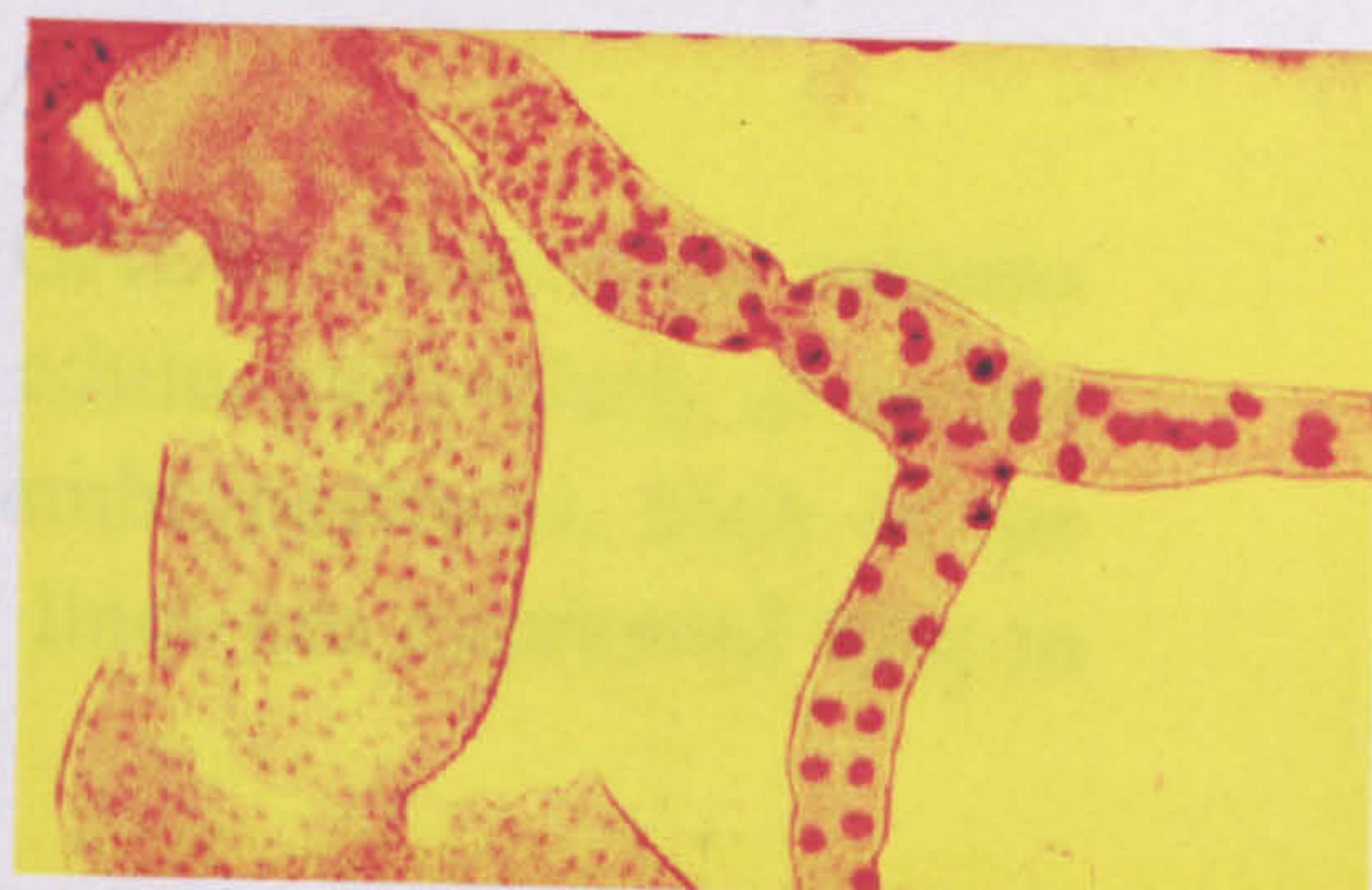
d)



e)



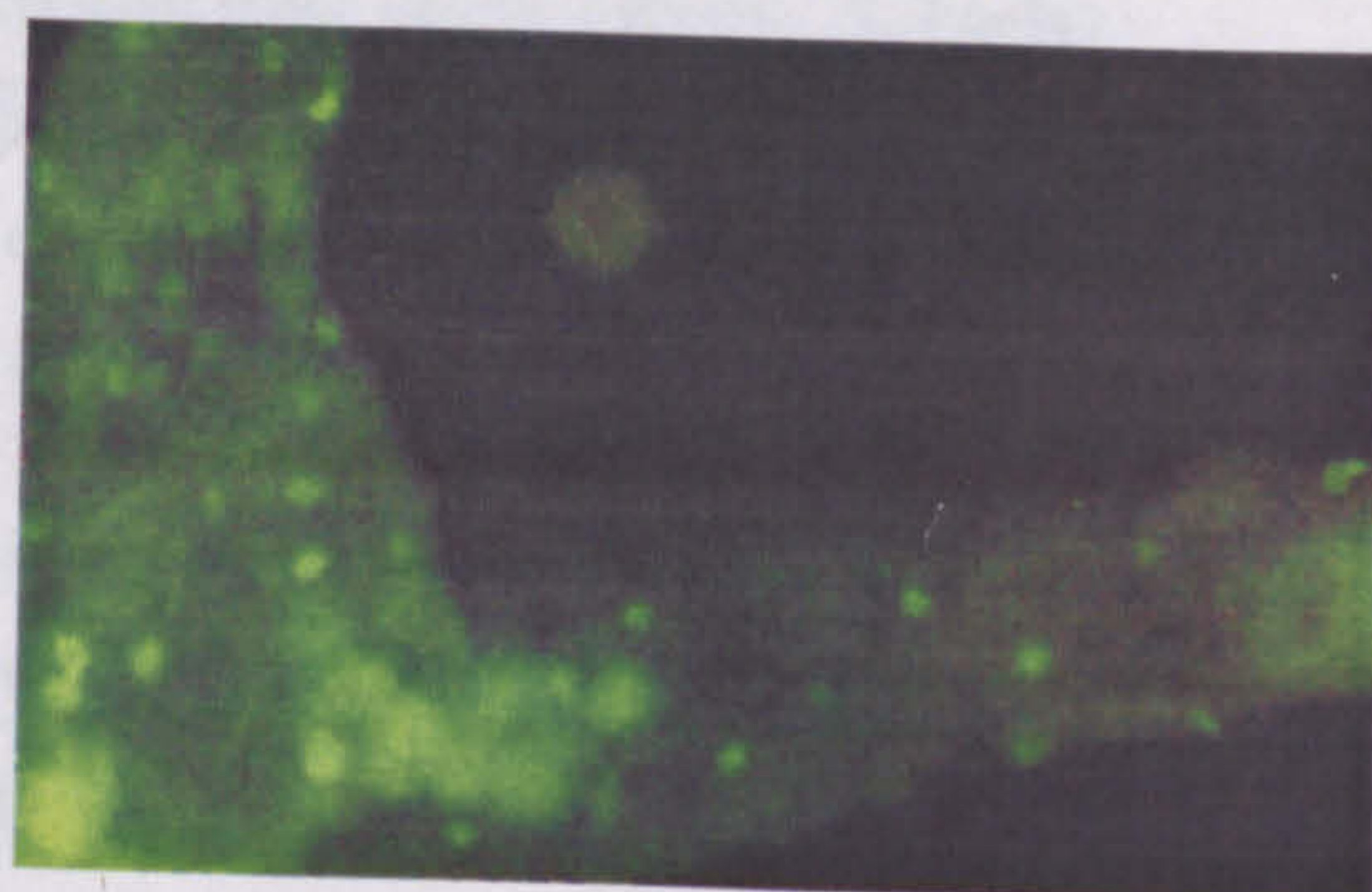
f)



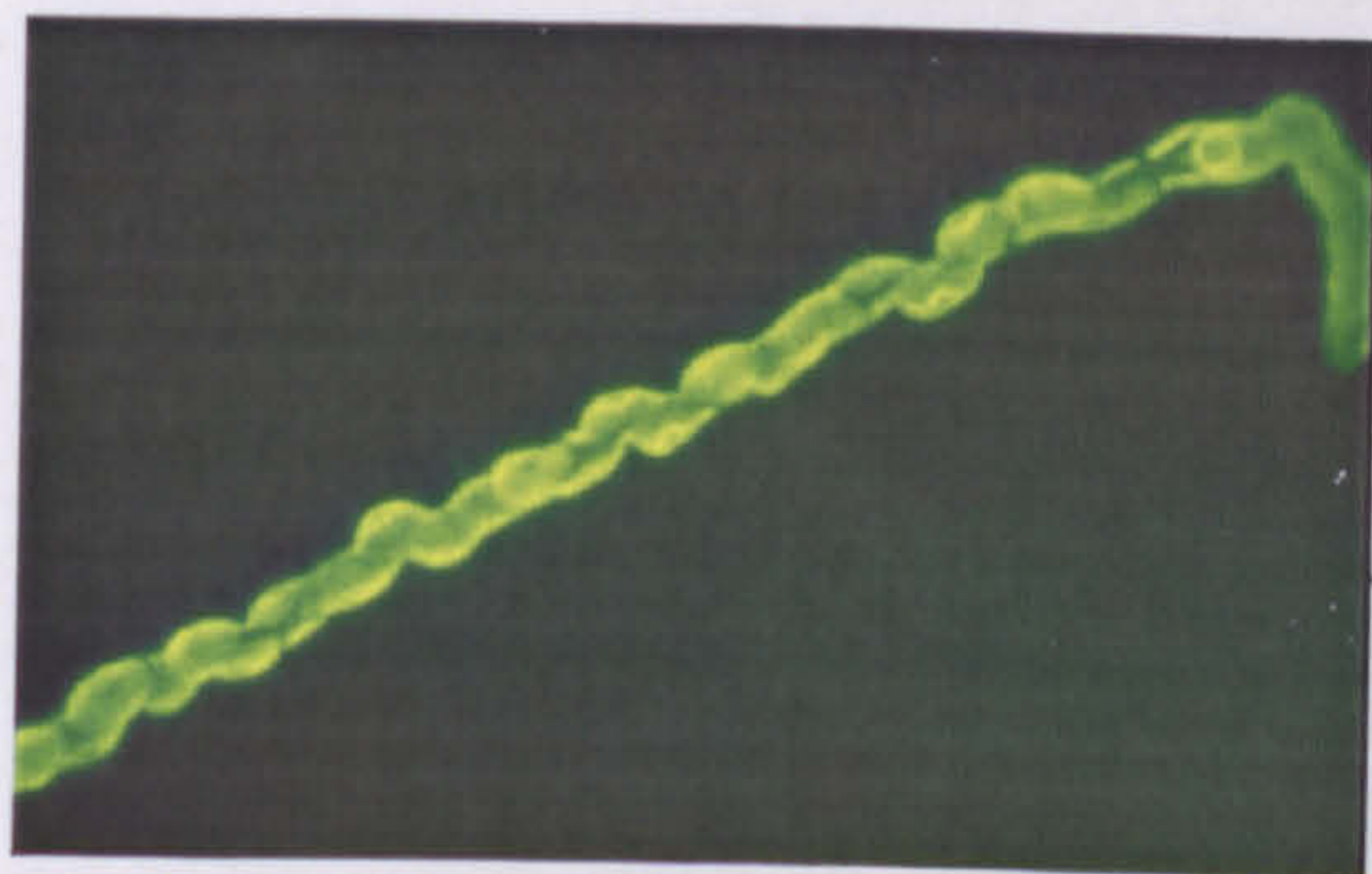
g)



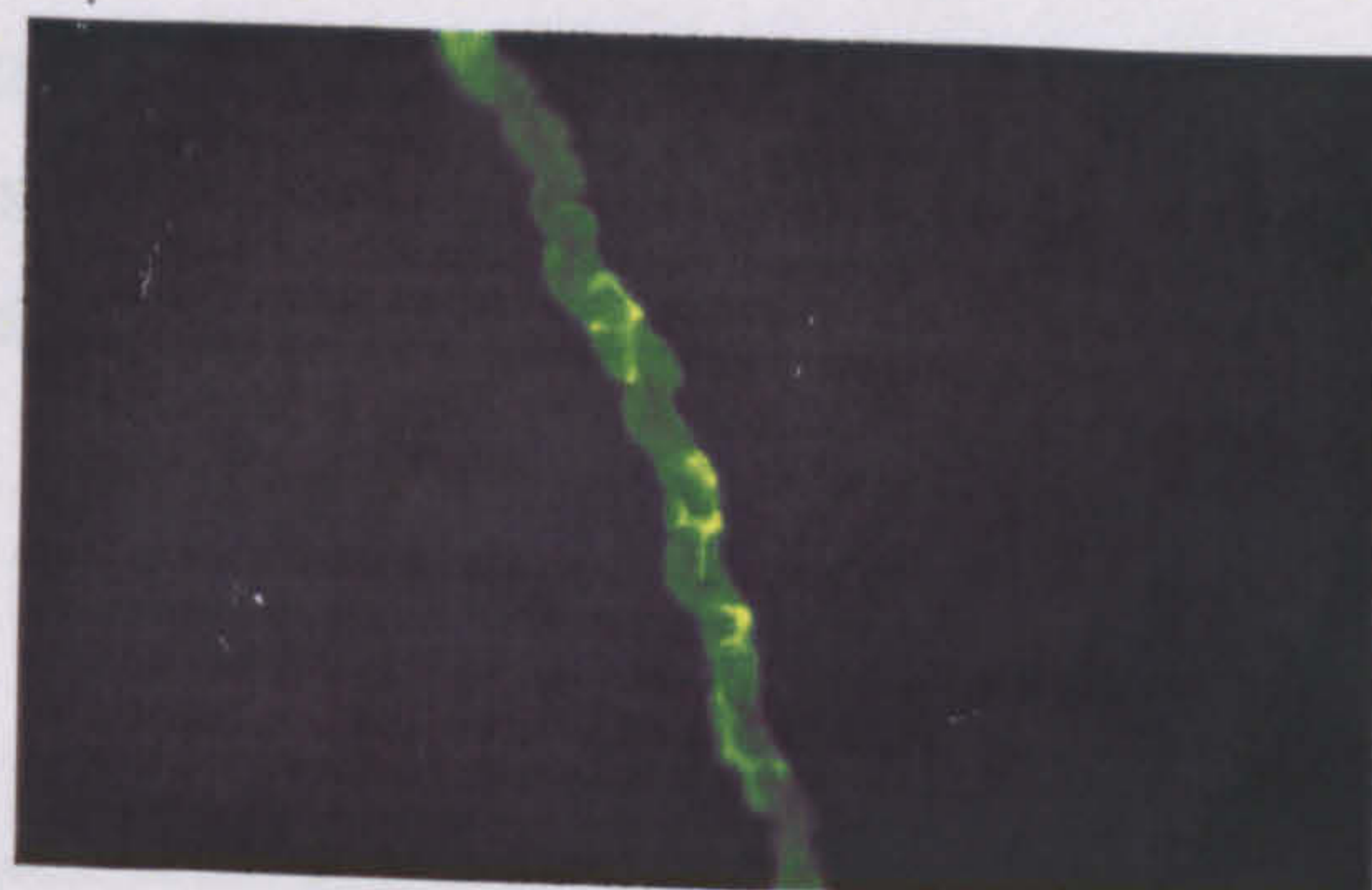
h)



i)



j)



3.6. Developmental aspects of stellate-cell specific P{GAL4} expressions.

Although the development of Malpighian tubules through a series of mechanisms was described broadly by many studies, what is known about cell-type specification in the mentioned tissue is very limited (Skaer, 1993). These findings constitute some clues about at least the approximate time of specification of cell-types in embryo and the significance of the stellate cells at subsequent developmental stages implying an important role for this subset of tubule cells.

The three P{GAL4} lines (C724, C710, C649) whose expression patterns in the adult tubule show stellate cell specificity were examined at all stages of development. Although there were some minor changes in expression patterns during development, the patterns were essentially similar from the earliest stages at which stellate cells could be identified.

3.6.1. Embryonal expression patterns of stellate cell-specific P{GAL4} lines.

In all the three stellate cell specific lines P{GAL4}, expression patterns were found to differ slightly from those of adults particularly at earlier embryonal stages. It appears that in early embryogenesis, each of the three distinct genetic loci marked by these lines are expressed first in different tissues.

Line C724 is initially expressed widely in ectodermal cells (**Figs. 3.7a,b**) before becoming confined to stellate cells (**Figs. 3.7c,d**). As can be seen in the late embryo (**Figure 3.7c,d**), stellate cells have probably already been specified, and GAL4 expression can be detected.

Line C649 reveals a segmentally iterated pattern in early embryos, which is strongly reminiscent of lateral sensory organ precursors of the peripheral nervous system (Jan and Jan, 1990).

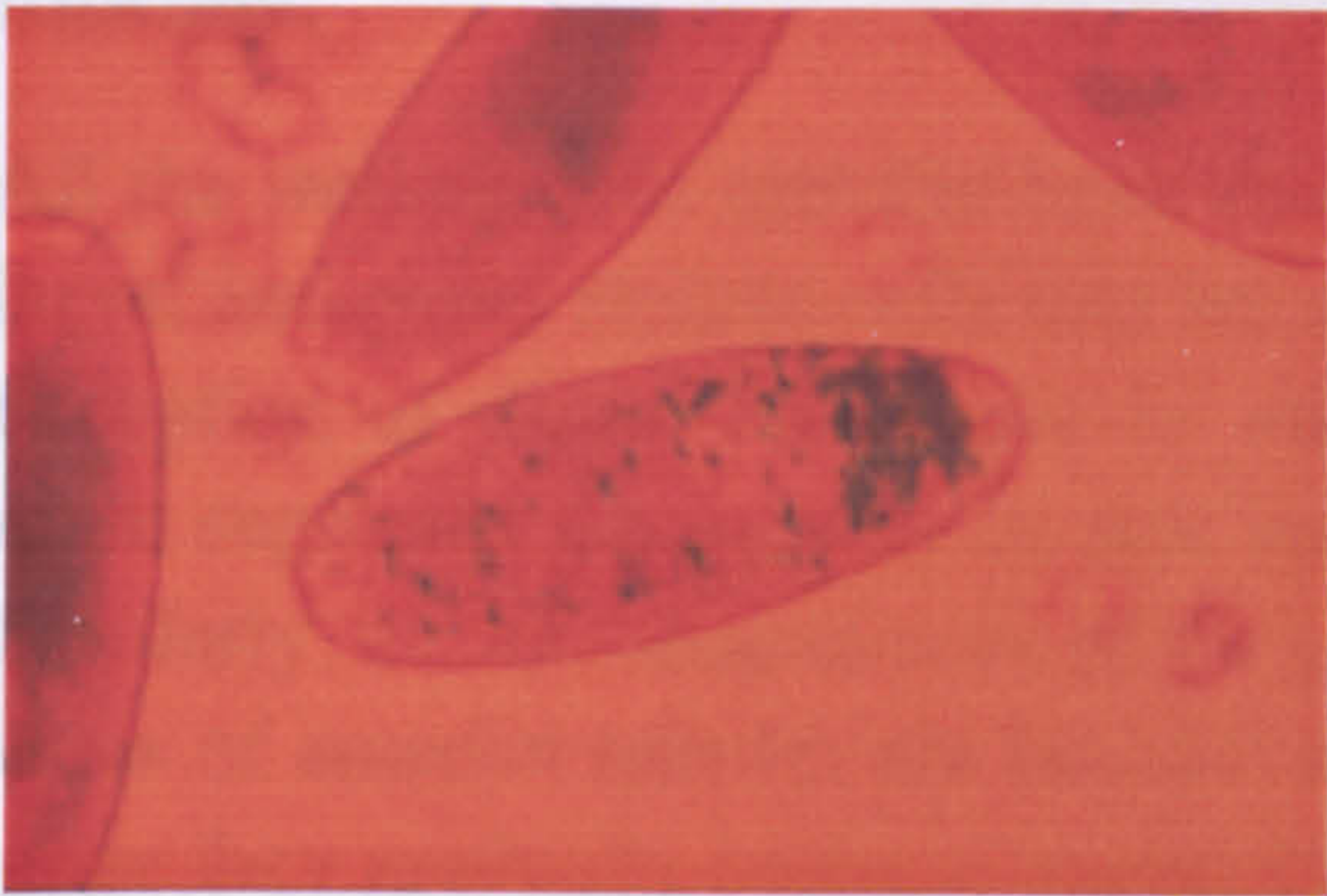
Due to the disadvantage of diffusion of *X-gal* during detection (**Figs 3.7. a, c, e, g**) pictures obtained with antibody staining against β -gal (**Figs. 3.7b,d,f,h**) are always given for comparison and better resolution.

Figure 3.7. Embryonal staining patterns of stellate cell specific lines

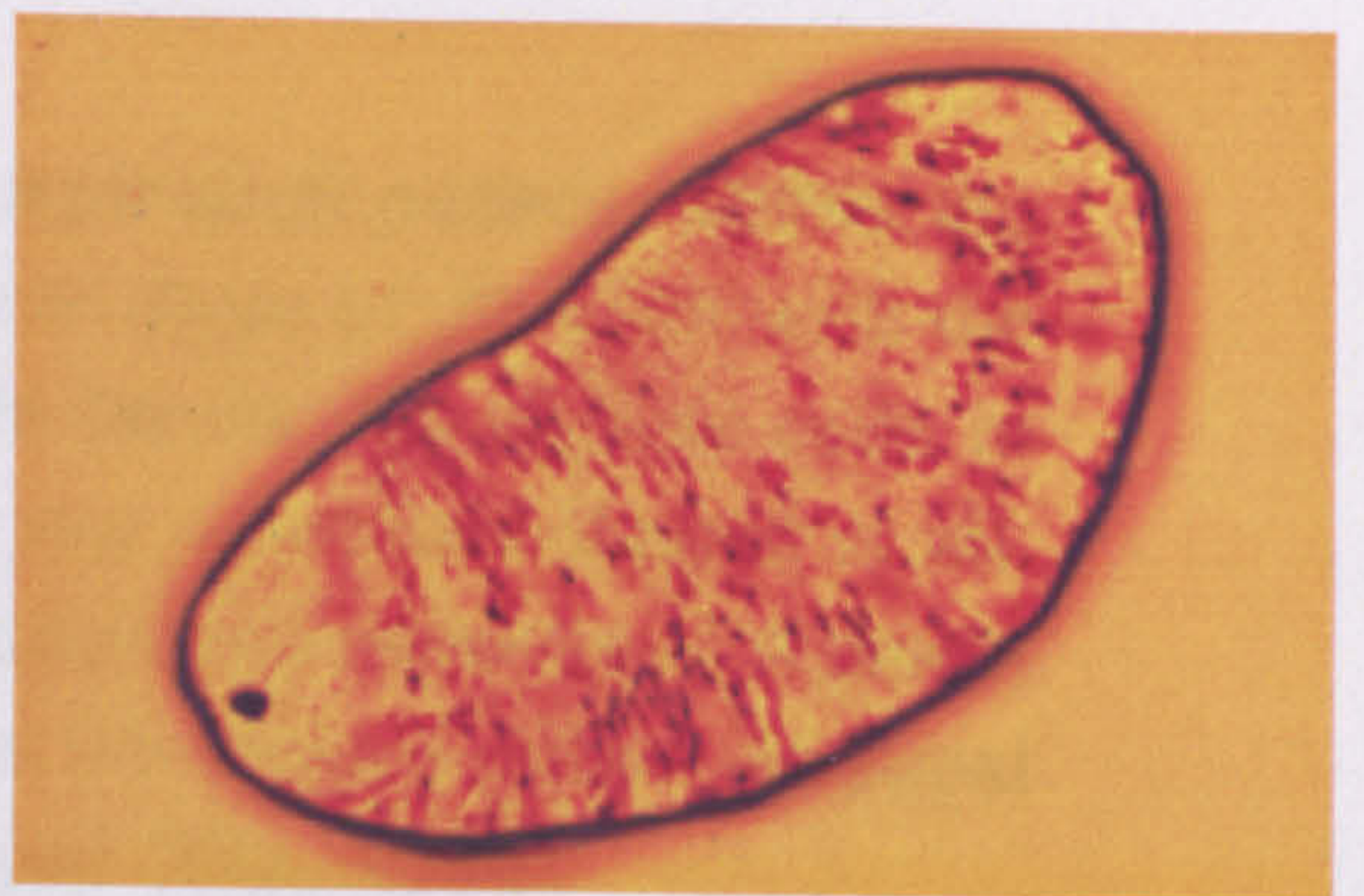
- a) Early embryonal staining in C724 with *X-gal*.
 - b) Early embryonal staining in C724 with widespread peripheral antibody staining, possibly in epidermis.
 - c) Late embryonal staining in C724 shows staining resolved to linear “beads”, suggesting the existence of stellate cells in embryo.
 - d) Antibody staining of hand-dissected C724 late embryo clearly shows the presence of stellate cells in tubules.
 - e) Late embryonal staining in C710 with *X-gal*.
 - f) Late embryonal staining in C710 with antibody staining. Staining is mainly in salivary glands.
 - g) Segmentally-iterated embryonal expression pattern in line C649 with *X-gal* staining
 - h) Antibody staining of line C649 expression pattern shows more detail i.e. presumably in PNS.
-

3.5.2. P(GAL4) expression in the larval and pupal midgut

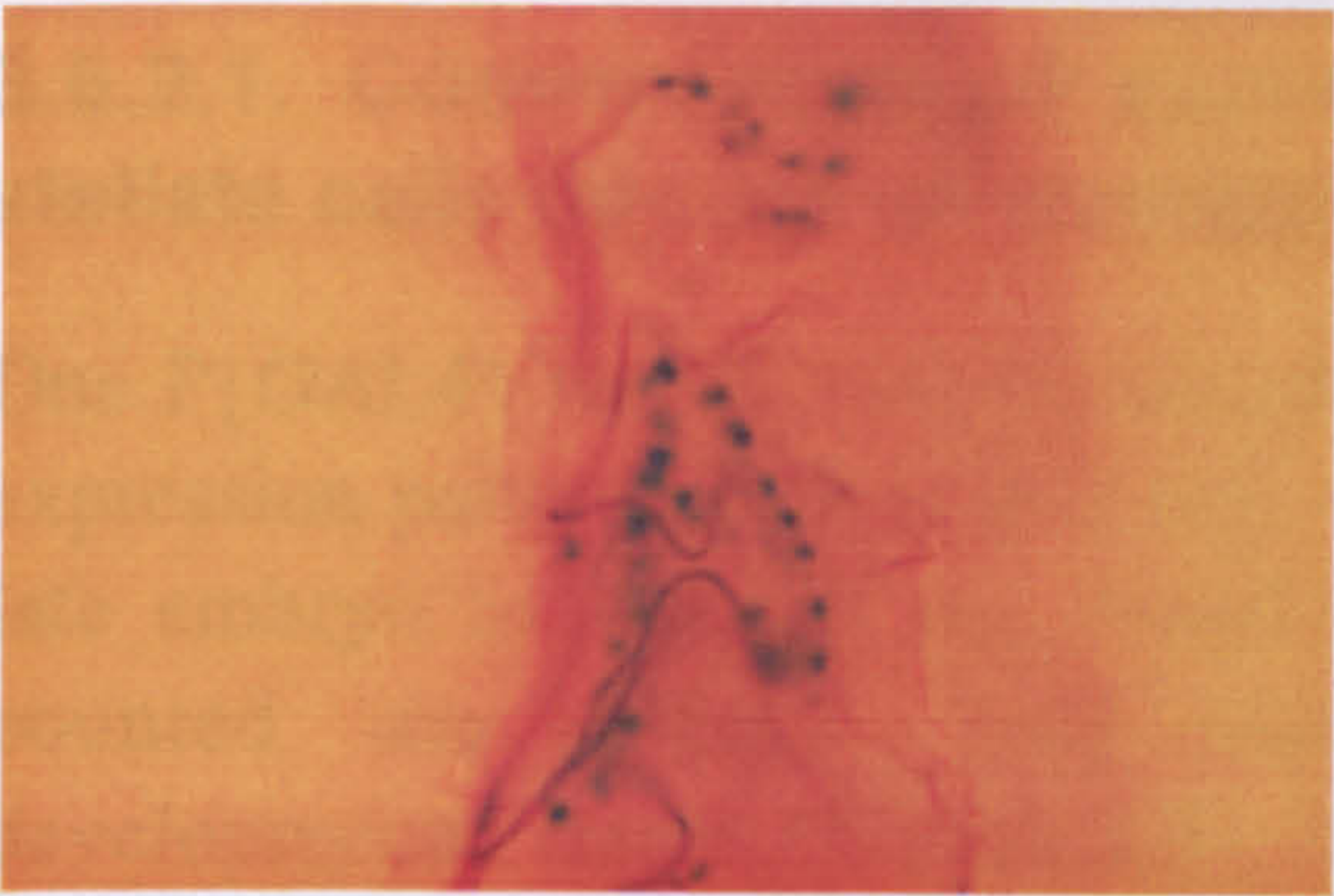
a)



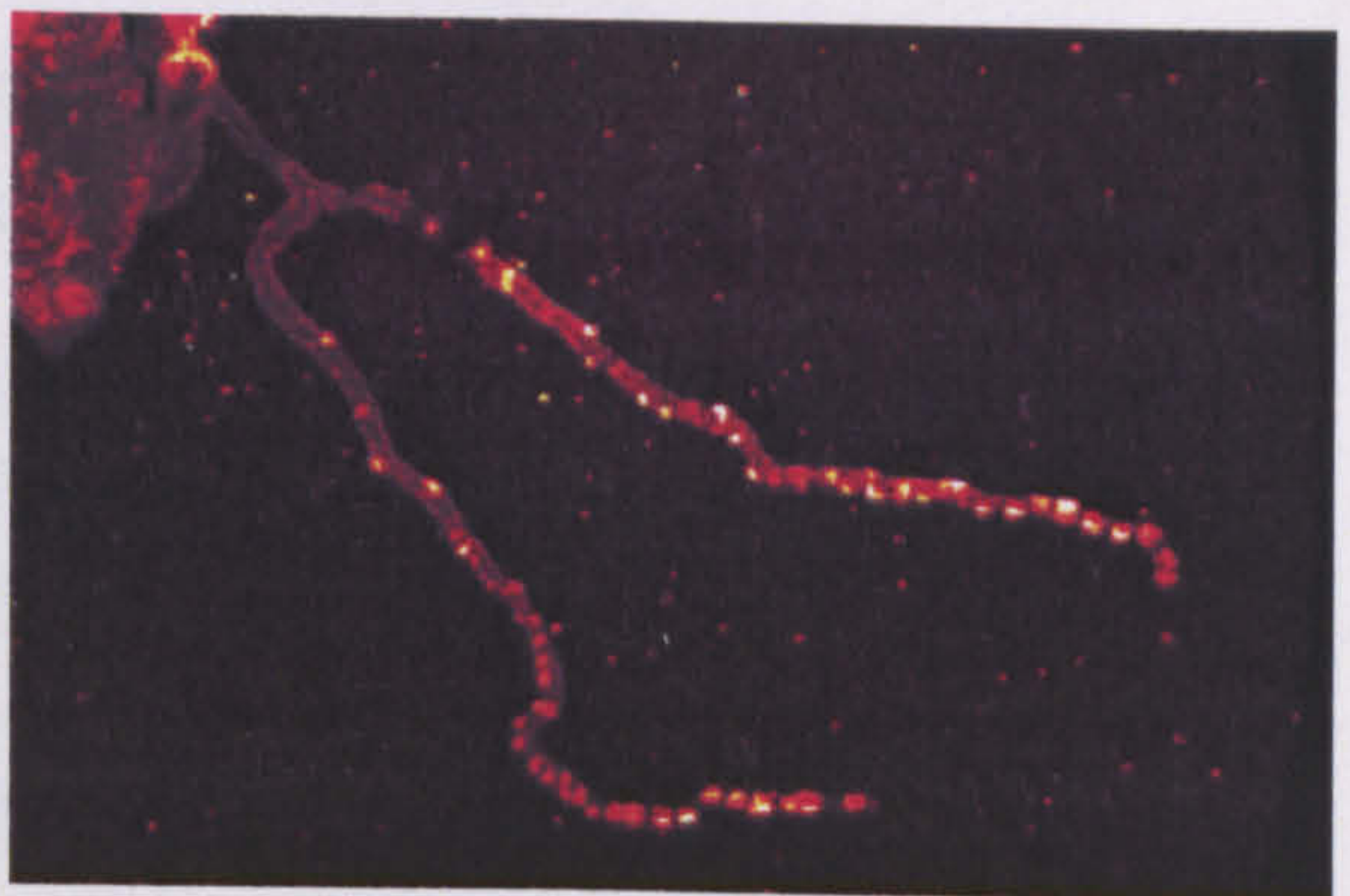
b)



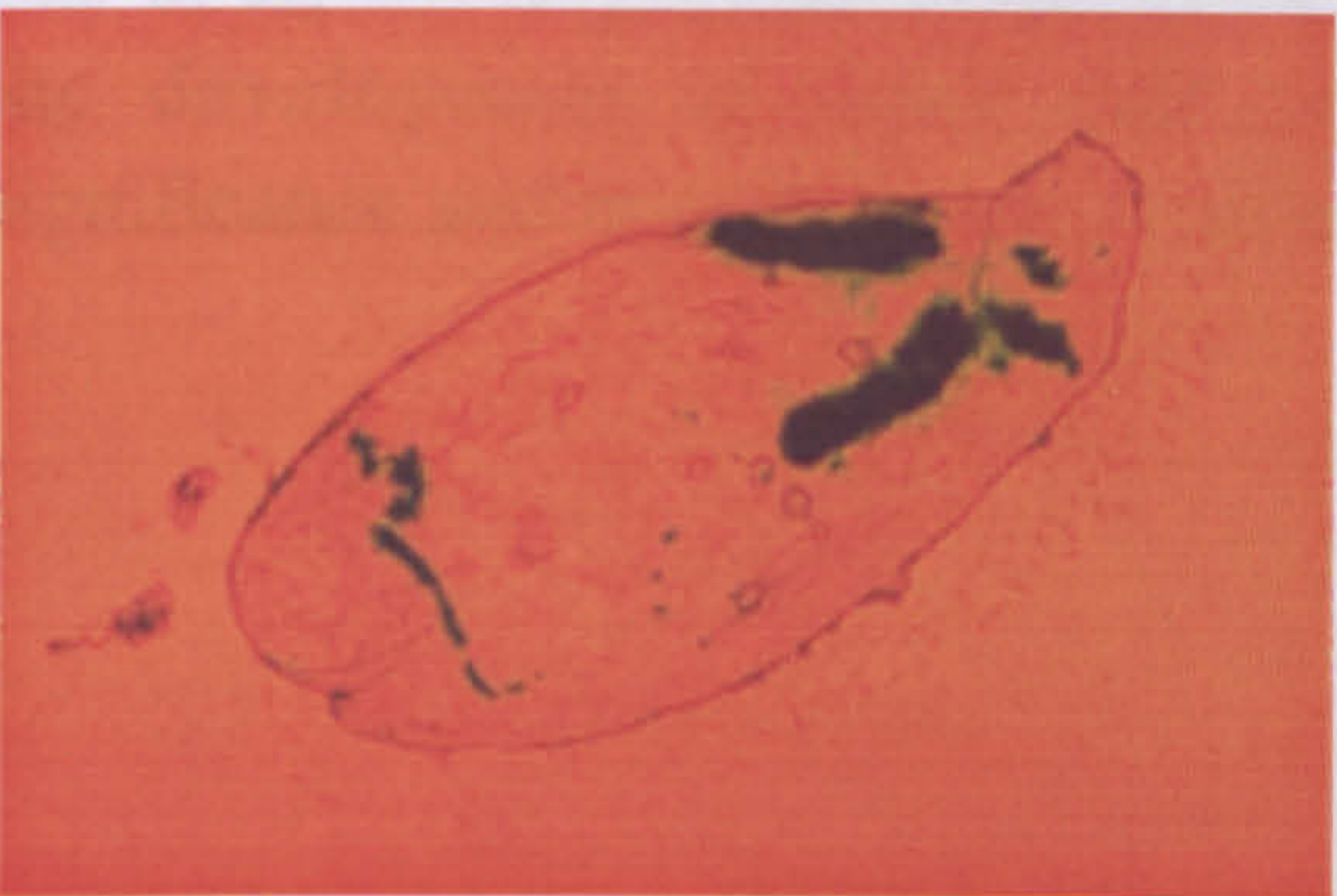
c)



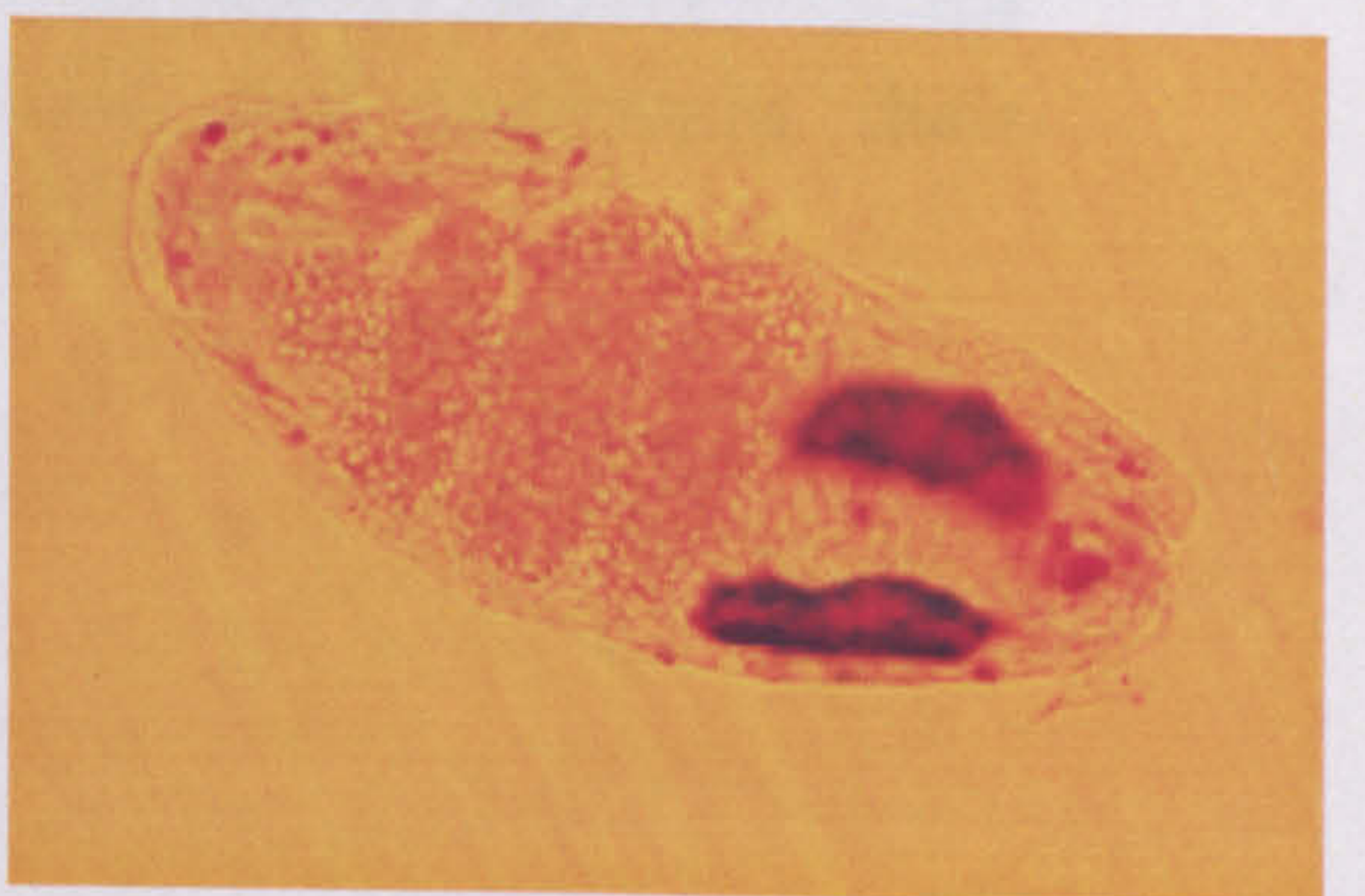
d)



e)



f)



g)



h)



3.6.2. P{GAL4} expression in the larval and pupal Malpighian tubules.

All the stellate cells specific lines showed expression patterns within the larval and pupal stellate cells that are similar to those of the adult with the exception of slight differences in shape. In larvae, the stellate cells in main segments are smaller in size and not star or stellate-shaped at all. Similarly, the bar-shaped cells of the adult initial and transitional segment areas of anterior tubules are broader and not bar-shaped either. At pupal stage they gradually begin to appear bar-shaped (**Fig. 3.25d**).

3.6.2.1. Conservation of expression and quantity in the stellate cells through all developmental stages.

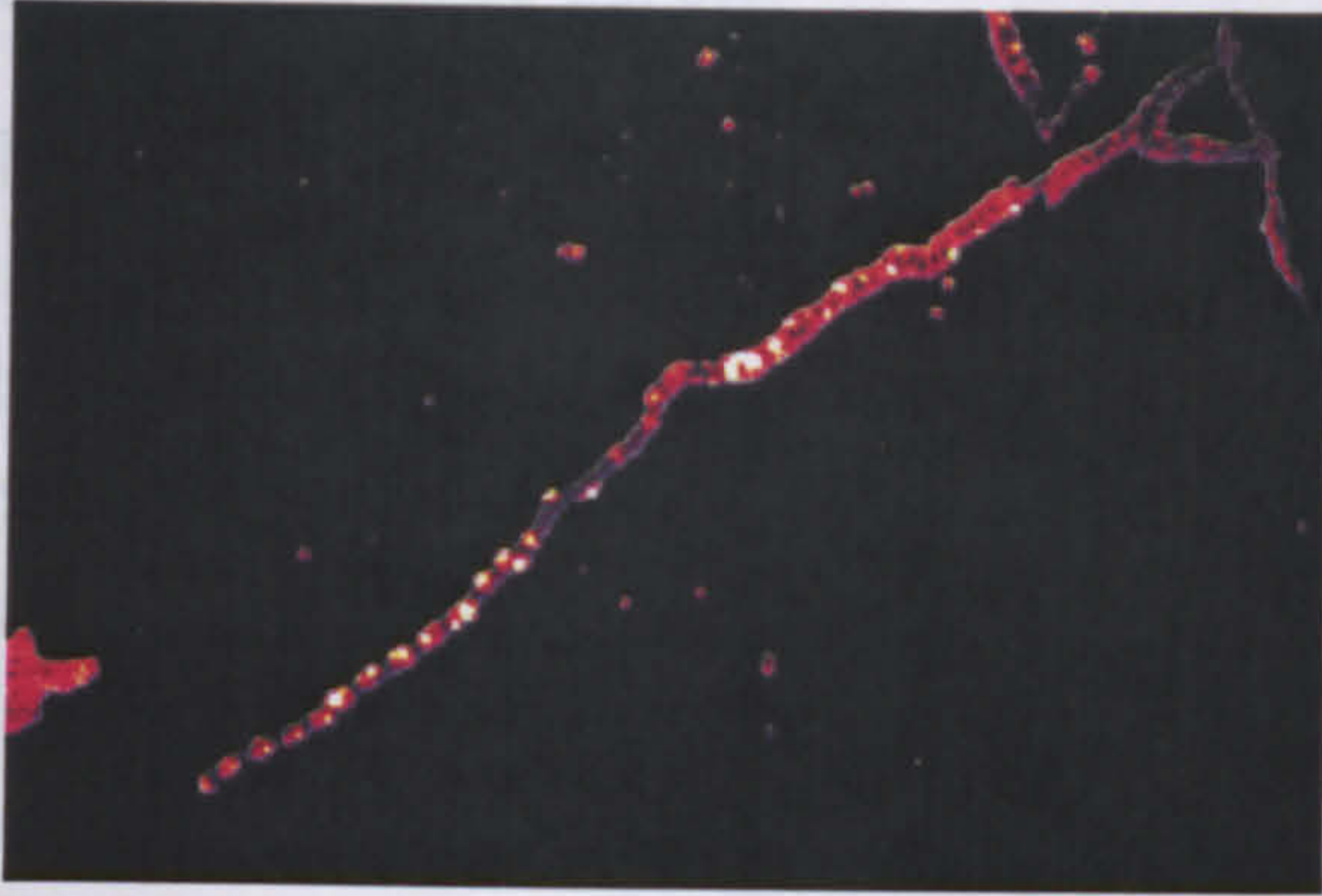
One P{GAL4} line (C724) possessed probably one of the most specific expression patterns seen in that only all stellate cells were marked from late embryo to adult. It was found that the numbers of stellate cells counted are strikingly very close to each other throughout all developmental stages (**Figure 3.8**). Here the results obtained from this line at all developmental stages have some remarkable implications for the development of these cell populations, and emphasize their significance and important role in tubule development.

Figure 3.8. The consistency of the stellate cells expression in line C724 through all developmental stages from first instar larva to the pupa.

- a) Stellate cells of anterior tubule in first instar larva.
 - b) Stellate cells of posterior tubule in first instar larva.
 - c) Stellate cells of anterior tubule in second instar larva.
 - d) Stellate cells of posterior tubule in second instar larva.
 - e) Stellate cells of anterior tubule in third instar larva.
 - f) Stellate cells of posterior tubule in third instar larva.
 - g) Stellate cells of anterior tubule in pupa.
 - h) Stellate cells of posterior tubule in pupa.
-

3.6.3. The diversity of P(OAL4) expression in the epidermal cells of third instar larvae

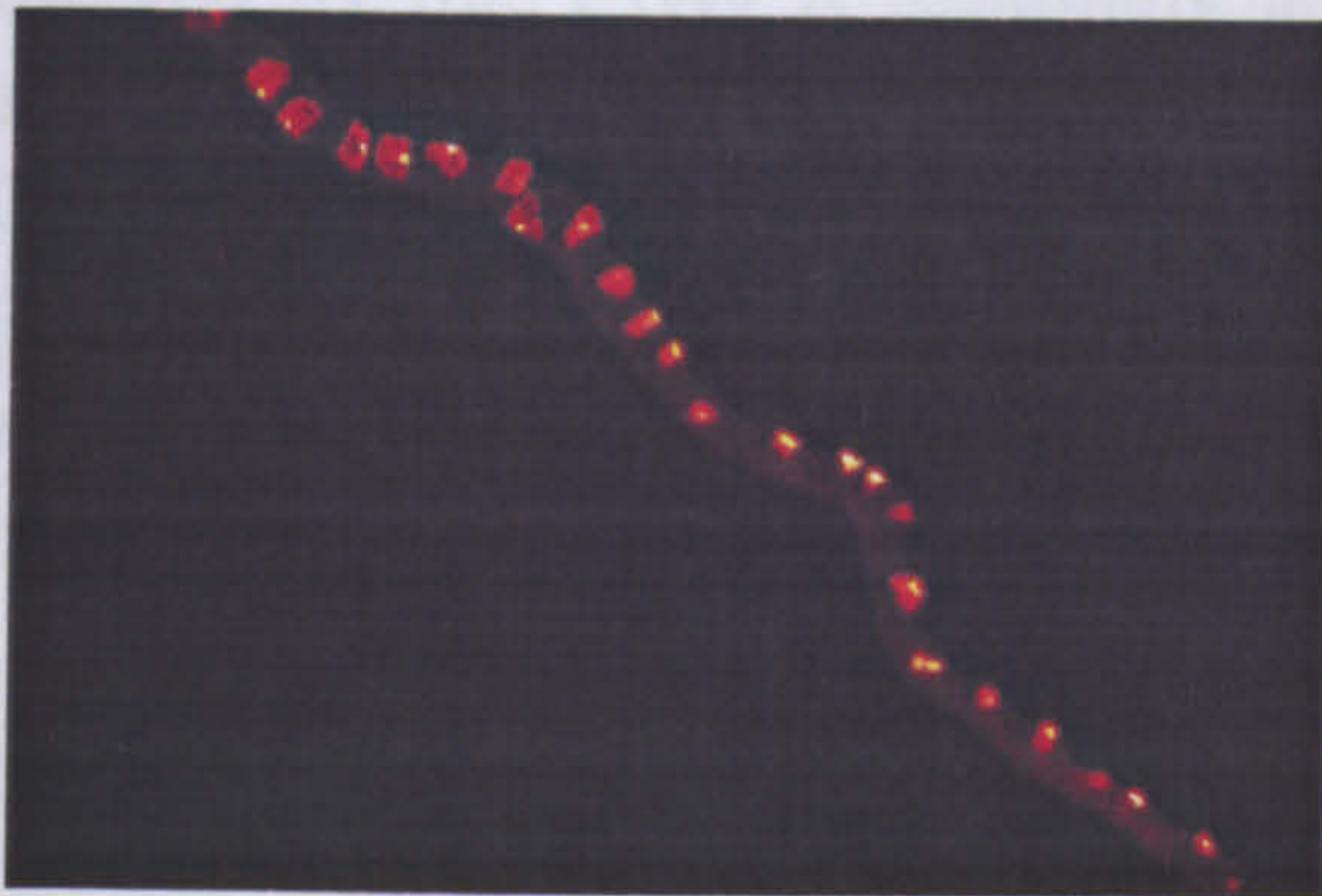
a)



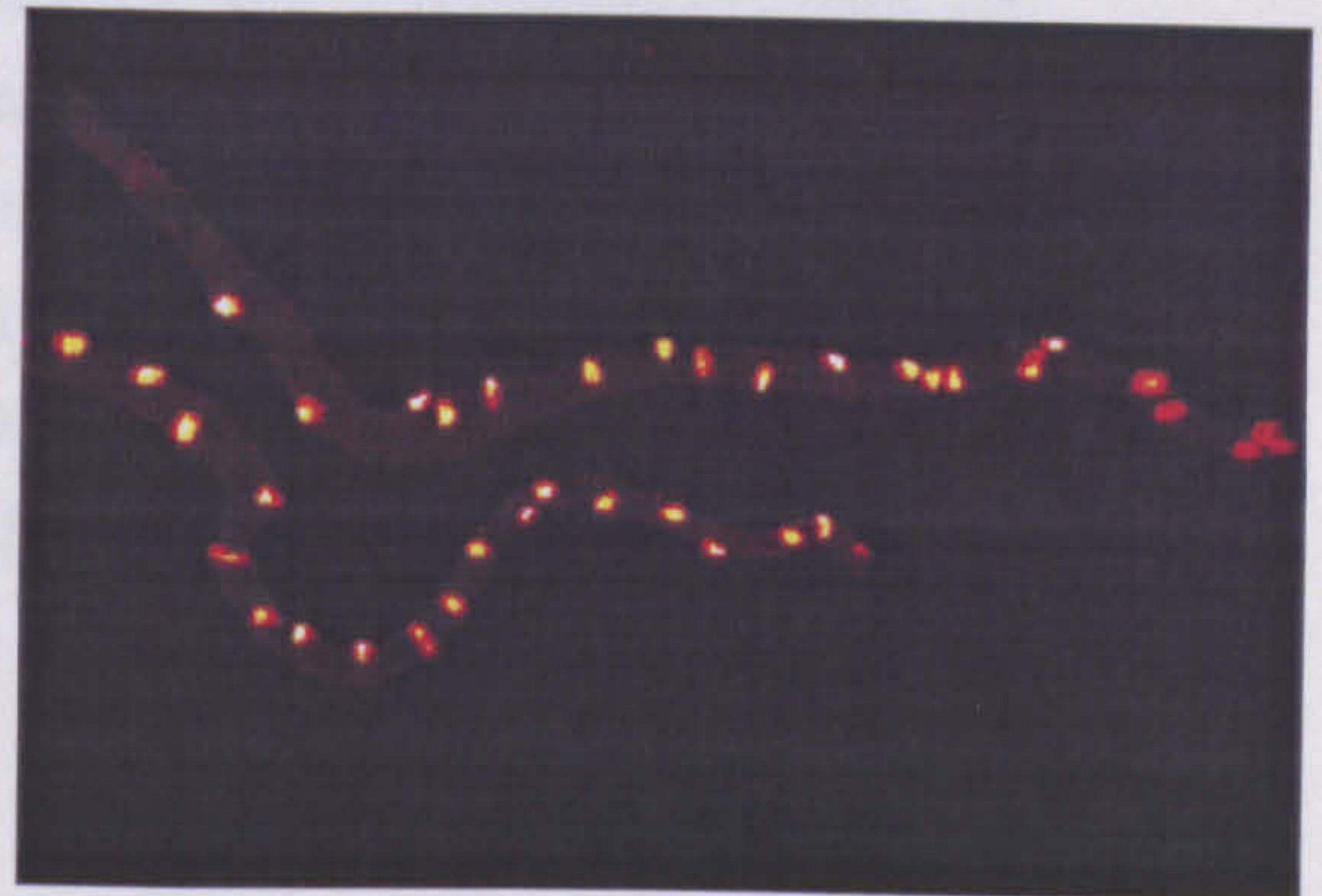
b)



c)



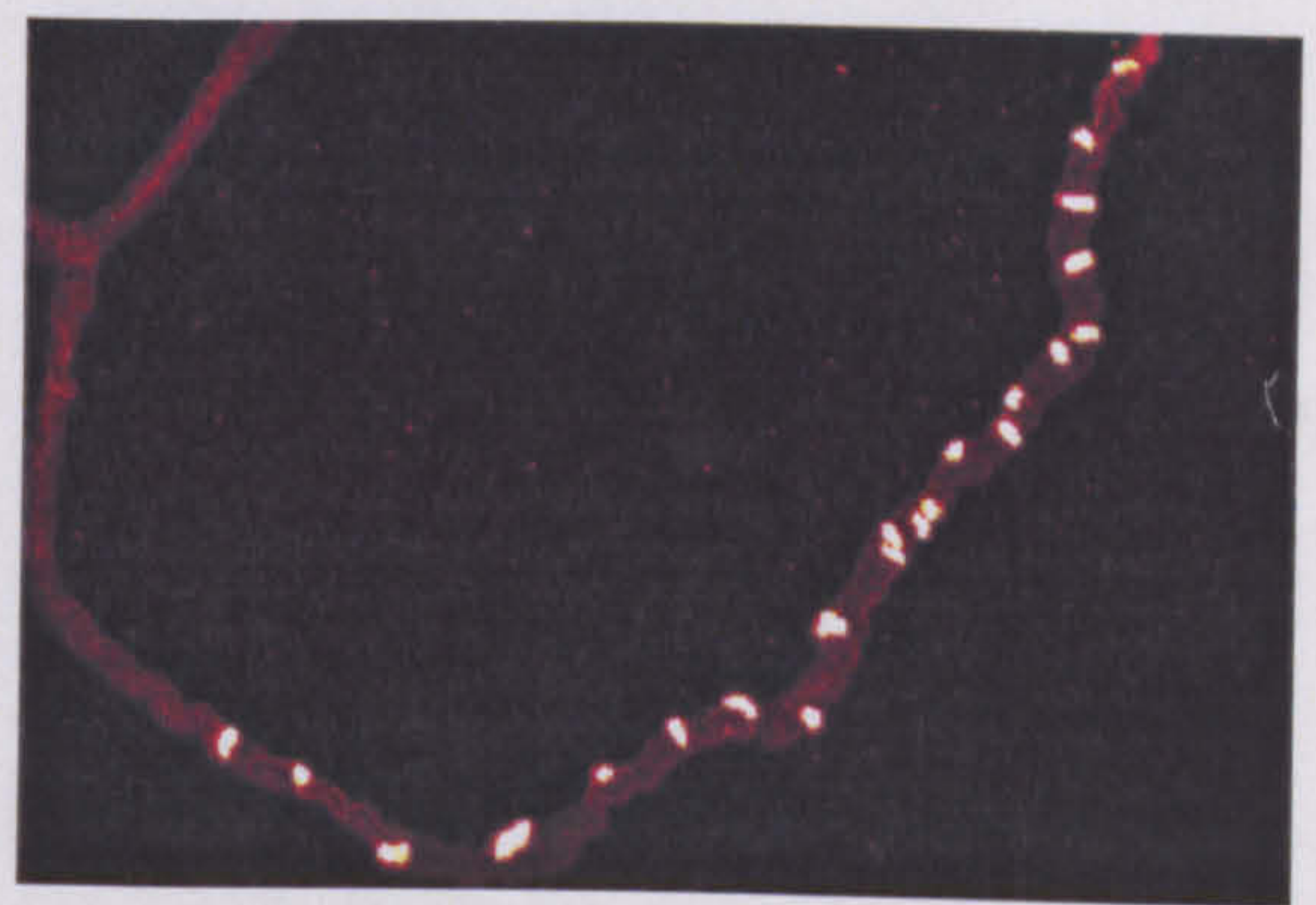
d)



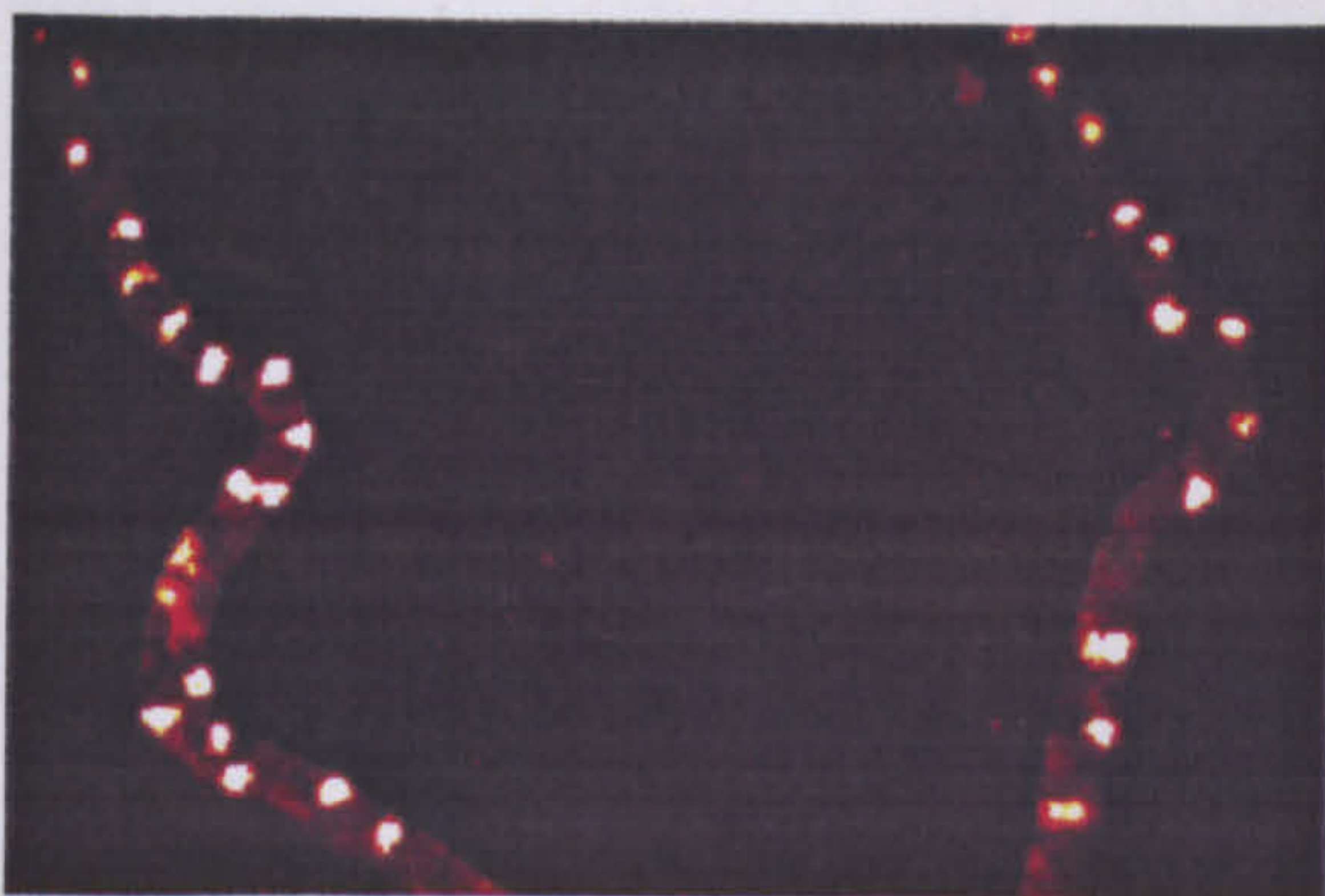
e)



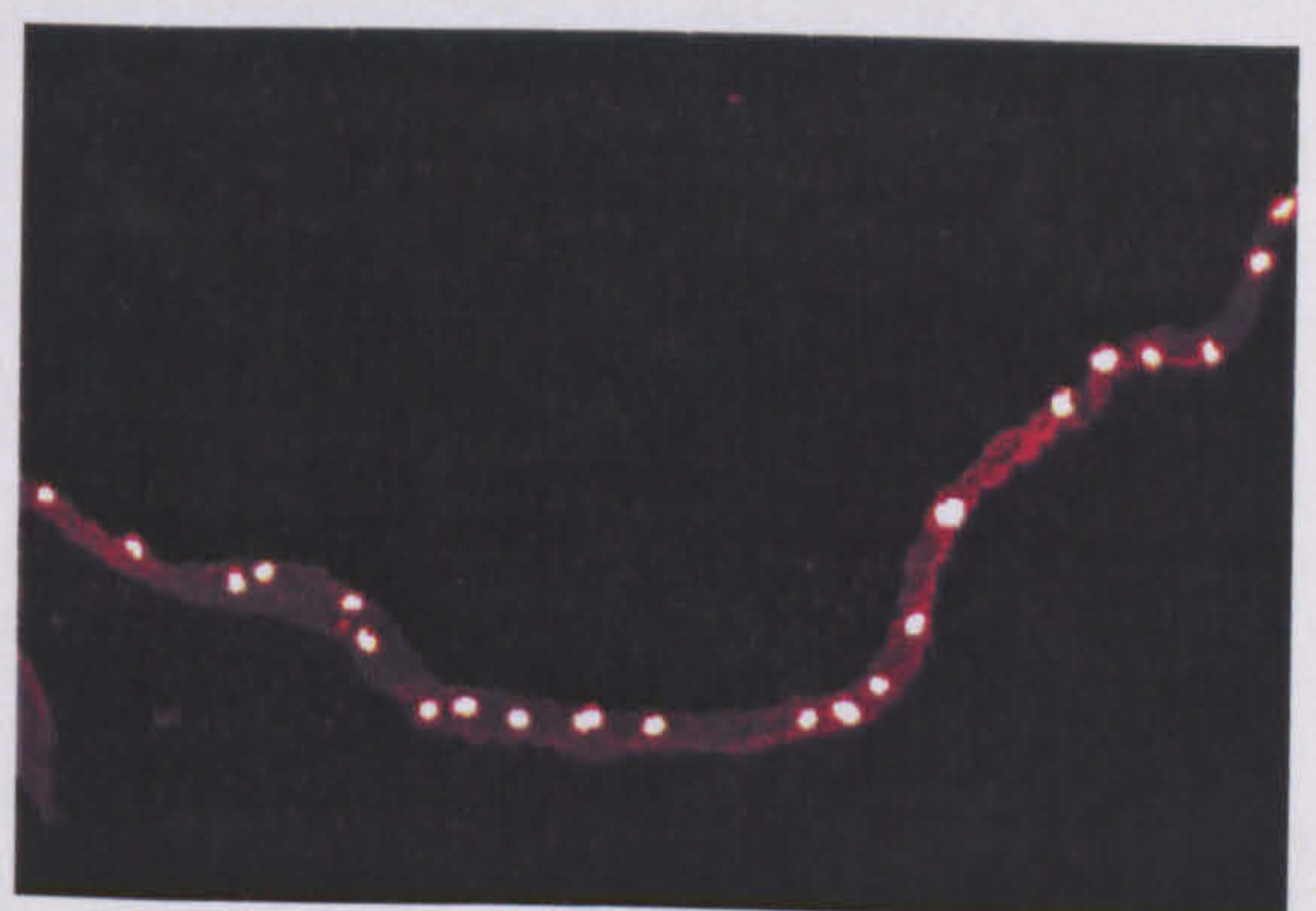
f)



g)



h)



3.6.3.The diversity of P{GAL4} expression in the stellate cells of third instar larva.

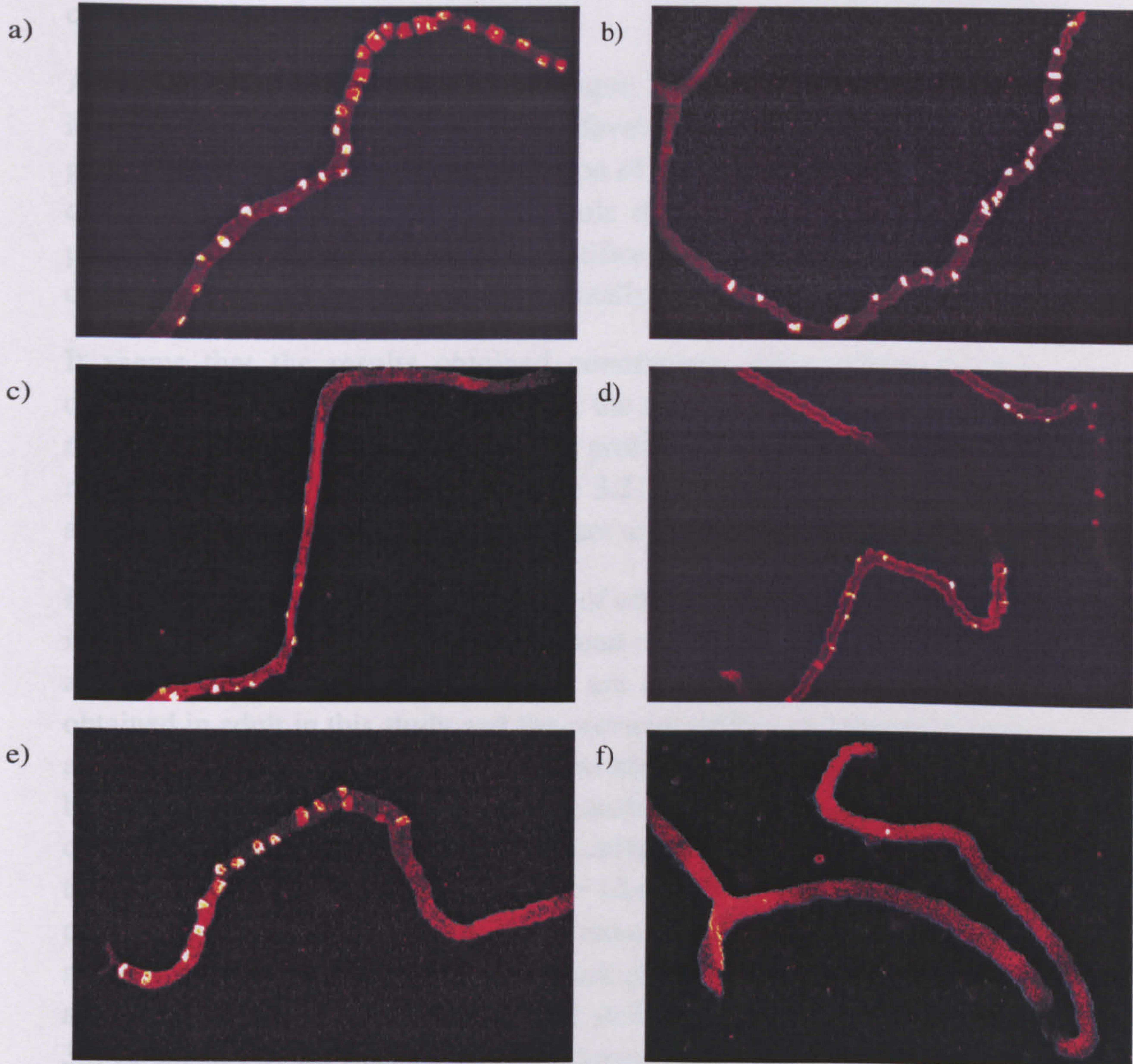
The diversity of P{GAL4} expression in the stellate cells of third instar larva reveals that stellate cells are subdivisible into two subgroups on the basis of P{GAL4} expression patterns. In line C724, P{GAL4} is expressed in all stellate cells at all developmental stages analysed (from embryo to adult). Whereas in line C649 expression is confined to only bar-shaped stellate cells of anterior tubule. No expression was observed at all in the stellate cells of posterior tubule. This suggests that the enhancer marked by line C649 is genuinely a marker of the secondary cells of anterior tubule initial and transitional segments. However, in line C710, GAL4 is expressed in the stellate cells of main segment of anterior tubules and posterior tubules at larval stage (3rd instar) although all stellate cells of adult express GAL4. This confirms that in some sense the two types of stellate cells could be different from each other.

Figure 3.9. The diversity of expression in the stellate cells of third instar larva suggests two types of stellate cells.

- a) In line C724 expression is in all stellate cells of anterior tubules.
 - b) In line C724 expression is in all stellate cells of posterior tubules.
 - c) In line C710 expression is only in all stellate cells in main segment of anterior tubules.
 - d) Line C710 marks all stellate cells of posterior tubules.
 - e) Line C649 distinguishes only the stellate cells of initial and transitional segment probably equivalent of that of the bar-shaped ones in the adult.
 - f) In line C649 there is no expression in the stellate cells of posterior tubule at all.
-

3.5.4. Quantitative developments of UASG-GFP expression

Quantification of larval and pupal nuclei with UASG-GFP expression in the central nervous system (CNS) was performed. Data obtained from UASG-GFP larvae with 100% penetrance are shown.



such nuclei in the region posterior to the 1st abdominal segment (Fig. 1.12, b) were 10.33±0.6 (n=20) principal cells in larva.

In addition, in line C50⁺, the number of UASG-GFP-positive cell nuclei in the lower tubule was 25±0.3 (n=20) for anterior tubule, 10.33±0.7 (n=18) for posterior tubule, giving a total of 35.33±0.6 (n=38). This corresponded closely with the value of 35.33±0.6 in the region where no nuclear cells were observed in line C50⁺, suggesting

3.6.4. Quantitative developmental analysis of domains of gene expression

Quantification of larval and pupal stellate cells and comparison of the data obtained from UASG-GFP lines with the previous results showed close agreement.

As an advantage of enhancer trap technique, the cell types can not only be identified in the adult and the other developmental stages, but also it gives another advantage of quantification of cell types and even domains quite accurately. As in the adult tubule domains and cell types it is possible to give a figure about the quantification of one of the major cells of the tubules, stellate cells, developmentally here.

It seems that the results obtained concerning cell numbers during development are quite close to those for the adult. So, in the light of these results it can be confirmed that after the proliferation is completed the cell numbers remain unchanged (see **Table 3.2**). The changes occur in other aspects such as shape, size or so on (Skaer and Martinez-Arias, 1992).

It has also been found that the numbers of ethidium-stained principal cells in some subregions and as well as the total cell numbers (**Figs 3.10 a, 4.1 and Table 3.4**) in third instar larvae are closely comparable to those obtained in adult in this study and the previous studies on embryo, larvae and adult (Janning *et al.*, 1986; Skaer and Martinez-Arias, 1992). For this, by counting such nuclei, it was possible to reconcile apparently complementary boundaries reported in different lines, and to prove that they were so close together as to be likely to be identical to those previously identified in the adult. For example, in 3rd instar larvae the number of ethidium-stained principal cell nuclei in the upper domain of anterior tubules marked by bar-shaped stellate cells (line C649, **Figure 3.3 e**) was 43.8 ± 0.6 (n=24); this corresponded closely with the sum of such nuclei in the region unstained in line C42, containing 45.2 ± 0.6 (n=20) principal cells in larva.

In addition, in line C507, the number of ethidium-stained principal cell nuclei in the lower tubule was 25 ± 0.5 (n=20) for anterior tubules 20.33 ± 0.7 (n=18) for posterior tubule giving an average of 22.84 ± 0.6 (n=38). This corresponded closely with the sum of such nuclei in the region where no stellate cells were observed in line C724, containing

24 ± 0.6 ($n=27$) principal cells both from anterior and posterior tubules altogether in third instar larvae.

It is also possible to reconcile functional and genetic maps in larval tubules. By counting the number of ethidium-stained principal cell nuclei in the rest of the tubule except lower tubule in line C507, containing 109 ± 2 ($n=11$) principal cells in anterior tubule of adult and 82 ± 1.4 ($n=19$) principal cells in posterior tubules respectively, this corresponded closely with the sum of such nuclei in the compartments unstained by alkaline phosphatase; in anterior tubule, 109 ± 2.7 ($n=13$), and in posterior tubule 82 ± 5.6 ($n=7$) (**Figure 3.5d**).

It is conspicuous that, although the measurements were derived from a large number of individuals from a number of lines which (by definition) have distinct genetic compositions, the numbers of cells both in the tubule overall, and the positions of genetic boundaries were practically invariant either between individuals or between lines. This suggests that tubule development is a remarkably robust and precise process, and allows the possibility that —for the first time— it will be feasible to study the development, placement, morphology and physiology of individual, identified cells within an epithelial model.

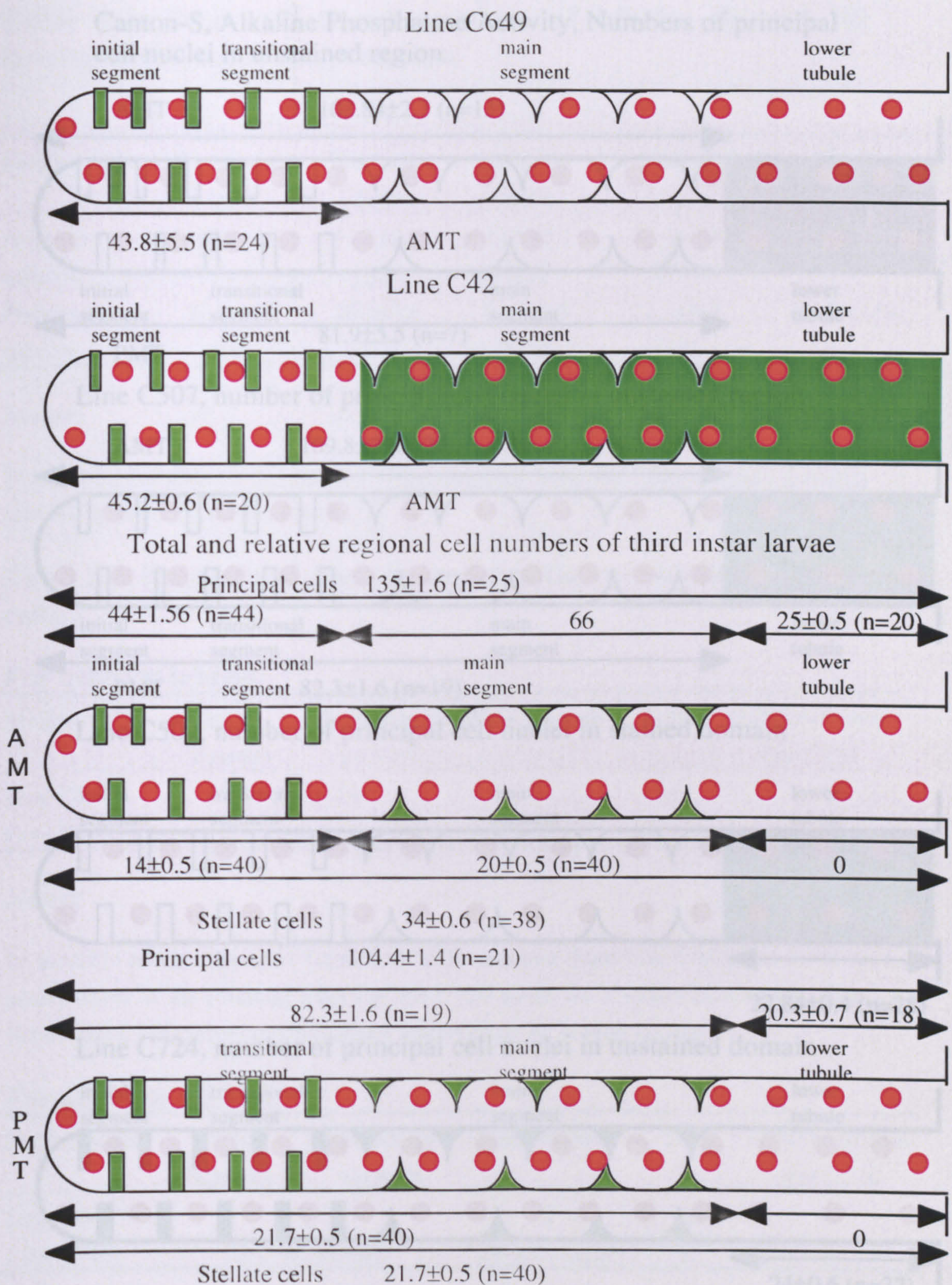
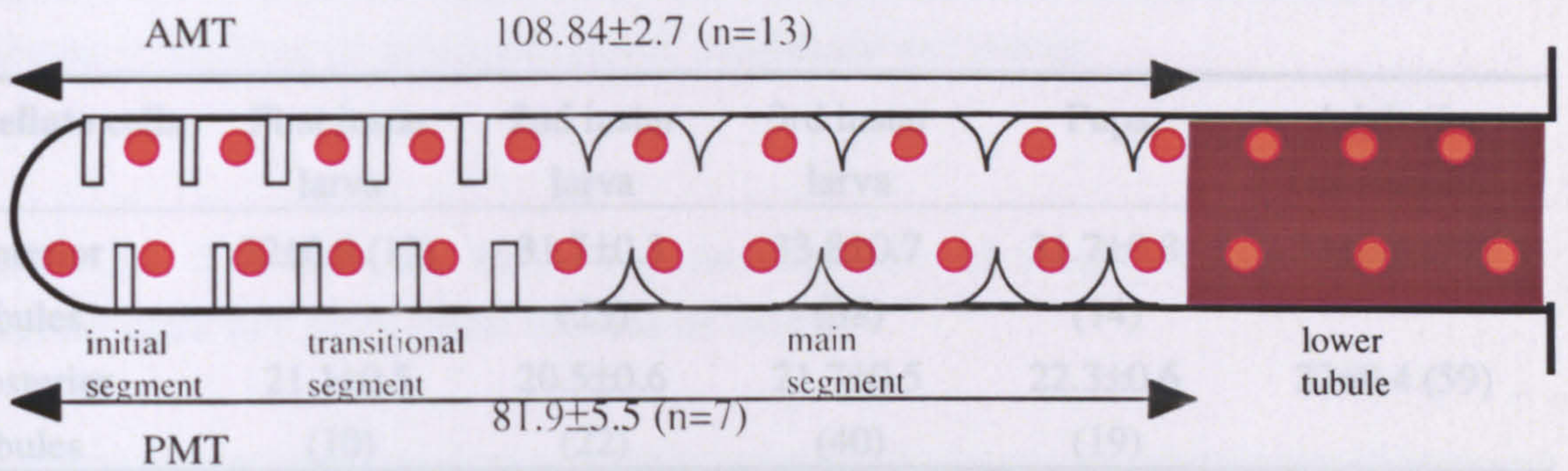


Figure 3.10 a. Reconciliation of complementary boundaries reported in different lines

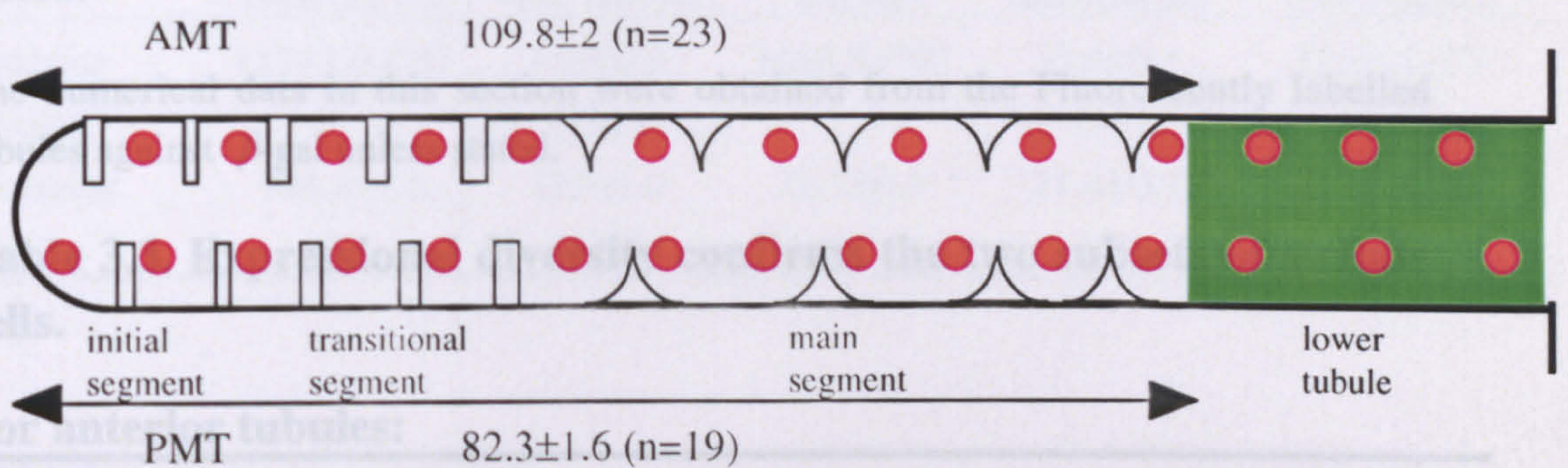
Complementary boundaries reported in different lines (upper two diagrams) and total and relative regional cell numbers in third instar larvae. Green denotes GAL4 expression pattern. Orange denotes ethidium stained principal cell nuclei.

Abbreviations: AMT: Anterior Malpighian tubule; PMT: Posterior Malpighian tubule.

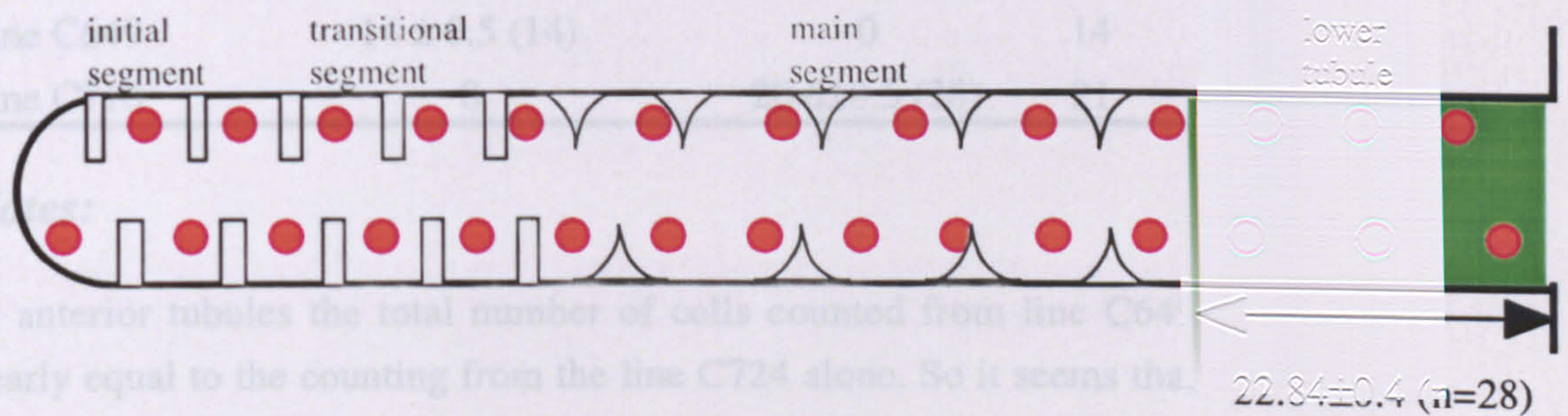
Canton-S, Alkaline Phosphatase Activity, Numbers of principal cell nuclei in unstained region



Line C507, number of principal cell nuclei in unstained region



Line C507, number of principal cell nuclei in stained domain



Line C724, number of principal cell nuclei in unstained domain

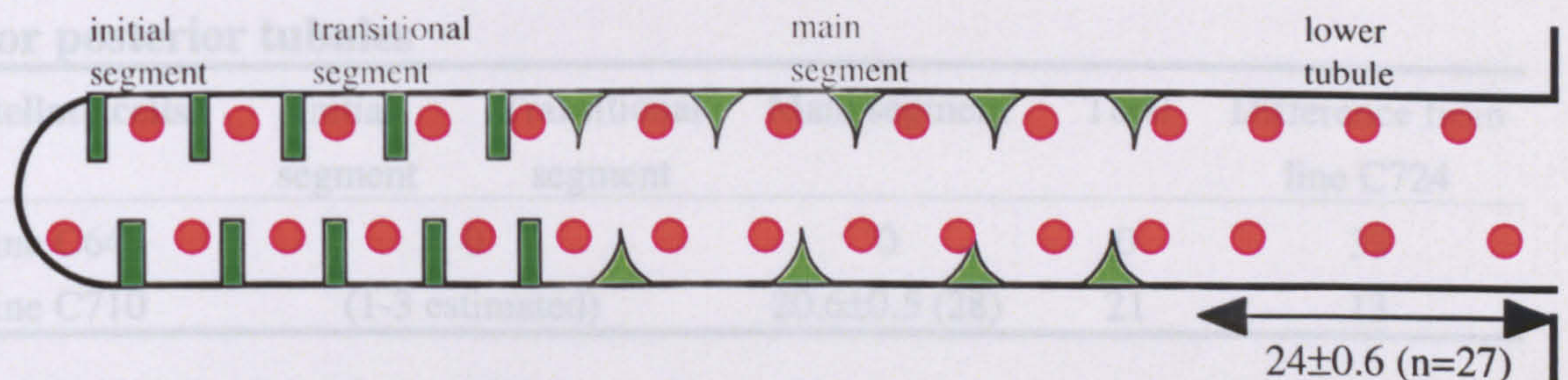


Figure 3.10 b Reconciliation of genetic and functional maps.

In third instar larvae, alkaline phosphatase activity maps closely to a region identical to that labelled by line C507 or that containing no stellate cells, was observed in line C724. Brown denotes alkaline phosphatase activity, green denotes GAL4 expression pattern.

Abbreviations: AMT: Anterior Malpighian tubule; PMT: Posterior Malpighian tubule

Table 3.2. Total stellate cell numbers of tubules through all developmental stages except embryo.

Stellate cells	First instar larva	2nd instar larva	3rd instar larva	Pupa	Adult (for comparison)
Anterior tubules	32±0.8 (12)	31.7±0.3 (23)	33.8±0.7 (38)	31.7±0.8 (14)	33±0.4 (77)
Posterior tubules	21.1±0.5 (10)	20.5±0.6 (22)	21.7±0.5 (40)	22.3±0.6 (19)	22±0.4 (59)

Notes:

The numerical data in this section were obtained from the Fluorescently labelled tubules against β -gal unless stated.

Table 3.3. Expressional diversity confirms the two subsets of stellate cells.

For anterior tubules:

Stellate cells	Initial segment	Transitional segment	Main segment	Total	Difference from line C724
Line C649	14 ± 0.5 (14)		0	14	19
Line C710		0	20.6±0.5 (28)	21	13

Notes:

In anterior tubules the total number of cells counted from line C649 and C710 is nearly equal to the counting from the line C724 alone. So it seems that the two lines (C649 and C710) label complementary subpopulations of type II cells.

For posterior tubules

Stellate cells	Initial segment	Transitional segment	Main segment	Total	Difference from line C724
Line C649		0	0	0	33
Line C710	(1-3 estimated)		20.6±0.5 (28)	21	13

Notes:

The total shown in the main segment section was due to the lack of marker in this line although it was from all subregions (main, transitional and initial)

Table 3.4. Larval (3rd instar) quantification of total principal and stellate cells are very close to that of adult.

Total cells	Principal cells (larva)	Principal cells (adult)	Stellate cells (larva)	Stellate* cells (larva)	Stellate cells (adult)
Anterior tubules	135±1.6 (25)	145±0.9 (123)	34±0.6 (38)	35.6±0.6 (30)	33±0.4 (77)
Posterior tubules	104.4±1.4 (21)	111±1.0 (120)	21.7±0.5 (40)	21.4±0.7 (18)	22±0.4 (59)

Notes:

* These data were obtained from the F1 generation of C724 X UAS_G-GFP cross.

Table 3.5. Summary of GAL4 directed GFP expression patterns in correlation with lacZ staining in the other sections

Line	Stage	LacZ staining	GFP expression	Expression pattern in the Malpighian tubule
C724	adult	Figs. 3.1 g-h Fig. 3.3 d Figs. 3.4 a-b Figs. 3.9a-b Fig. 3.24 b-d	Fig. 3.12, Fig. 3.13	All type II cells (stellate and bar-shaped)
C724	Larva (3)	Fig. 3.4 c Figs. 3.8 e-f Figs. 3.9 a-b Figs. 3.24 f-g	Fig. 3.18	All type II cells (stellate and bar-shaped)
C710	adult	Fig. 3.3 c Fig. 3.3 h Fig. 3.24 a	Fig. 3.14	All type II cells (stellate and bar-shaped)
C710	Larva (3)	Figs. 3.9 c-d Fig. 3.24 h	Fig. 3.19	Type II cells (only stellate-shaped ones)
C649	adult	Fig. 3.3 e	Fig. 3.15	Bar-shaped cells
C649	Larva (3)	Fig. 3.2 i Figs. 3.9 e-f	Fig. 3.20	Bar-shaped cells upper ureter
C42	adult	3.16 g	Fig. 3.16	All type I (principal) cells except those in the initial and transitional segments, bar-shaped cells not stellate-saped ones in the main segment
C42	Larva (3)	?	Fig. 3.22	All type I cells except those in the initial and transitional segments, bar-shaped cells not stellate-saped ones in the main segment and only upper ureter not lower ureter
C709	adult	Fig. 3.2 a Fig. 3.4 g	Fig. 3.17	Type I cells except in the miniature initial and transitional segments of PMT; but no type II cells
C507	Larva (3)	?	Fig. 3.21	Lower tubule and upper ureter; probably type I cells of initial and transitional segments not type II cells

3.7. Other Results

3.7.1. Vital detection of GAL4 expression

The value of the genetic mapping data presented here would be greatly increased if it were possible to visualise genetic compartments in the living tubule. This would allow precise physiological analysis.

GAL4 expression can be detected vitally either using lipophilic β -galactosidase substrates, or by simply crossing with UASG-Green Florescent Protein (GFP) lines which has insertion either on chromosome II or III.

In this study, the latter choice has been found extensively useful, quick and time-saving. Indeed, it would probably have been the method of choice, had it been available at the outset of this work.

It was also found that ethidium labels the Malpighian tubules. As an advantage of GAL4-UAS system, accordingly it has become possible to design experiments on identified cells in a living epithelium, and by doing so, it was possible to show that the compartment marked genetically by bar-shaped cells was precisely complementary to that marked physiologically by ethidium labelling (**Figs 3.5 c and 3.23**).

In this part, the GFP vital staining technique was used to extend the previous results obtained by histochemical techniques. With this technique, it proved straightforward to provide a detailed characterisation of the main lines as outlined on the facing page in **table 3.5**.

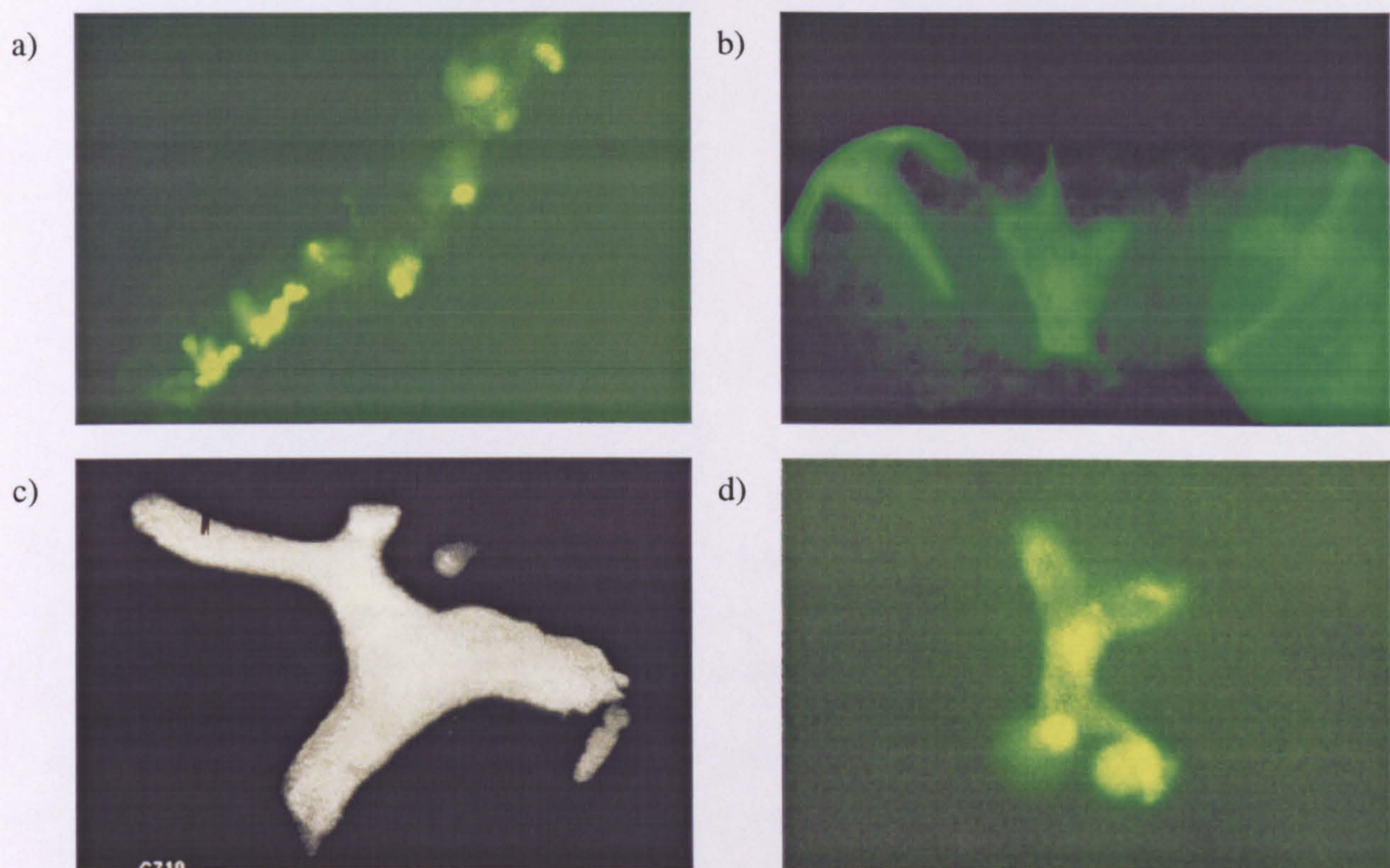
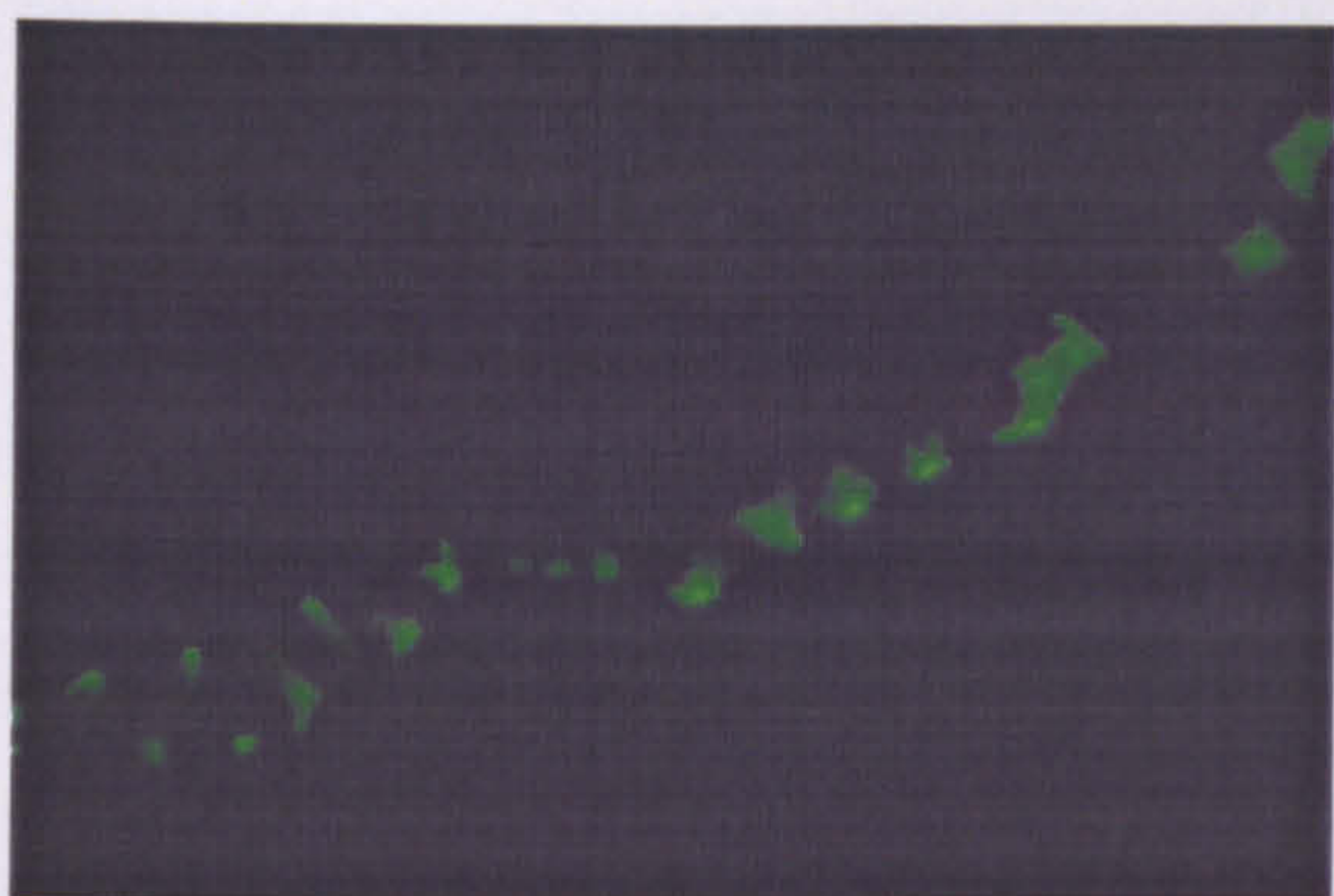


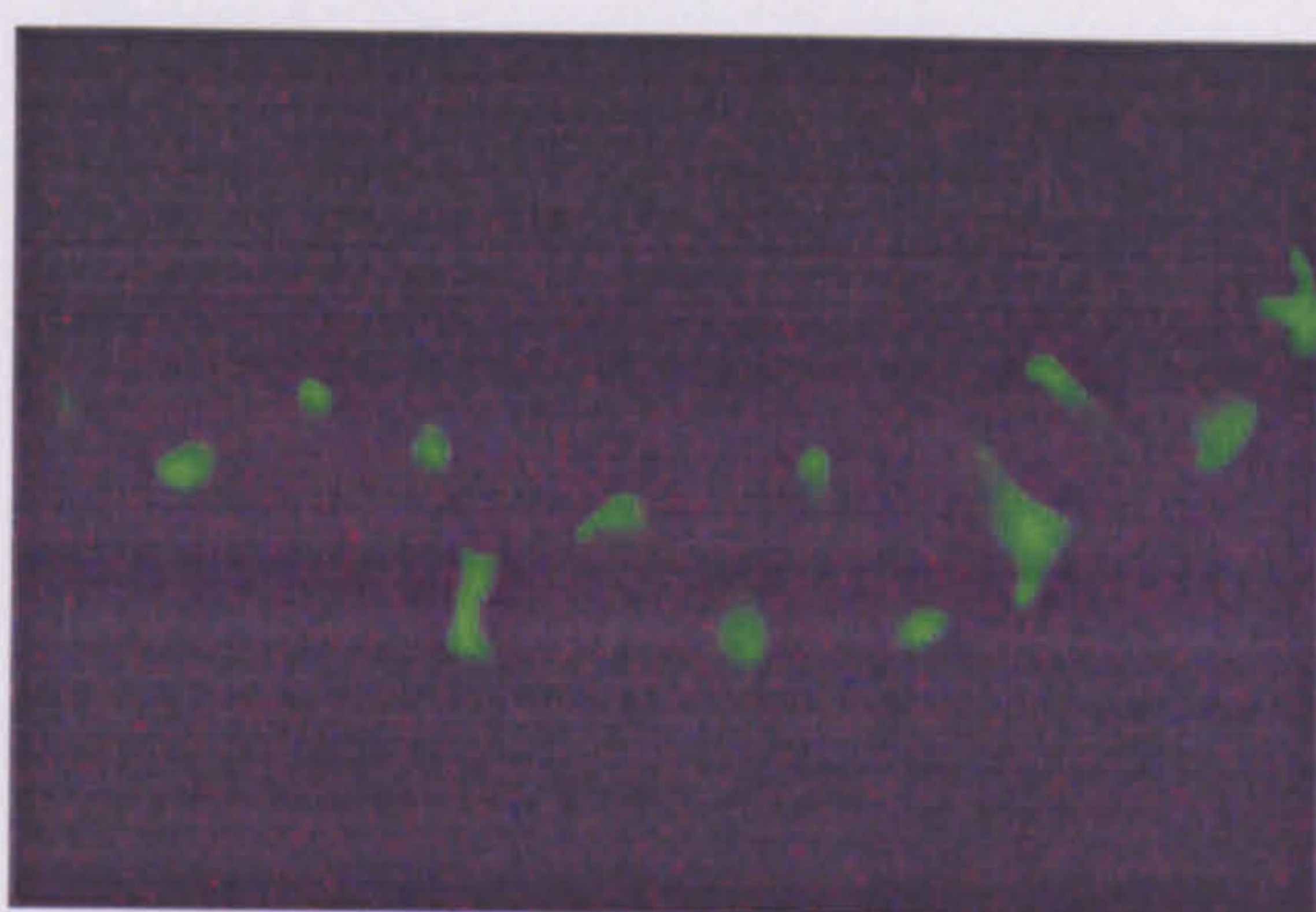
Figure 3.11. Vital staining of stellate cells using suitable substrates and isolation of one single stellate cell by collagenase.

- (a) Some stellate cells stained for β -gal vitally using lipophilic substrate.
- (b) Three stellate cells stained for β -gal vitally using lipophilic substrate in higher resolution.
- (c) One stellate cell stained vitally in highest resolution.
- (d) A single stellate cell isolated by collagenase.

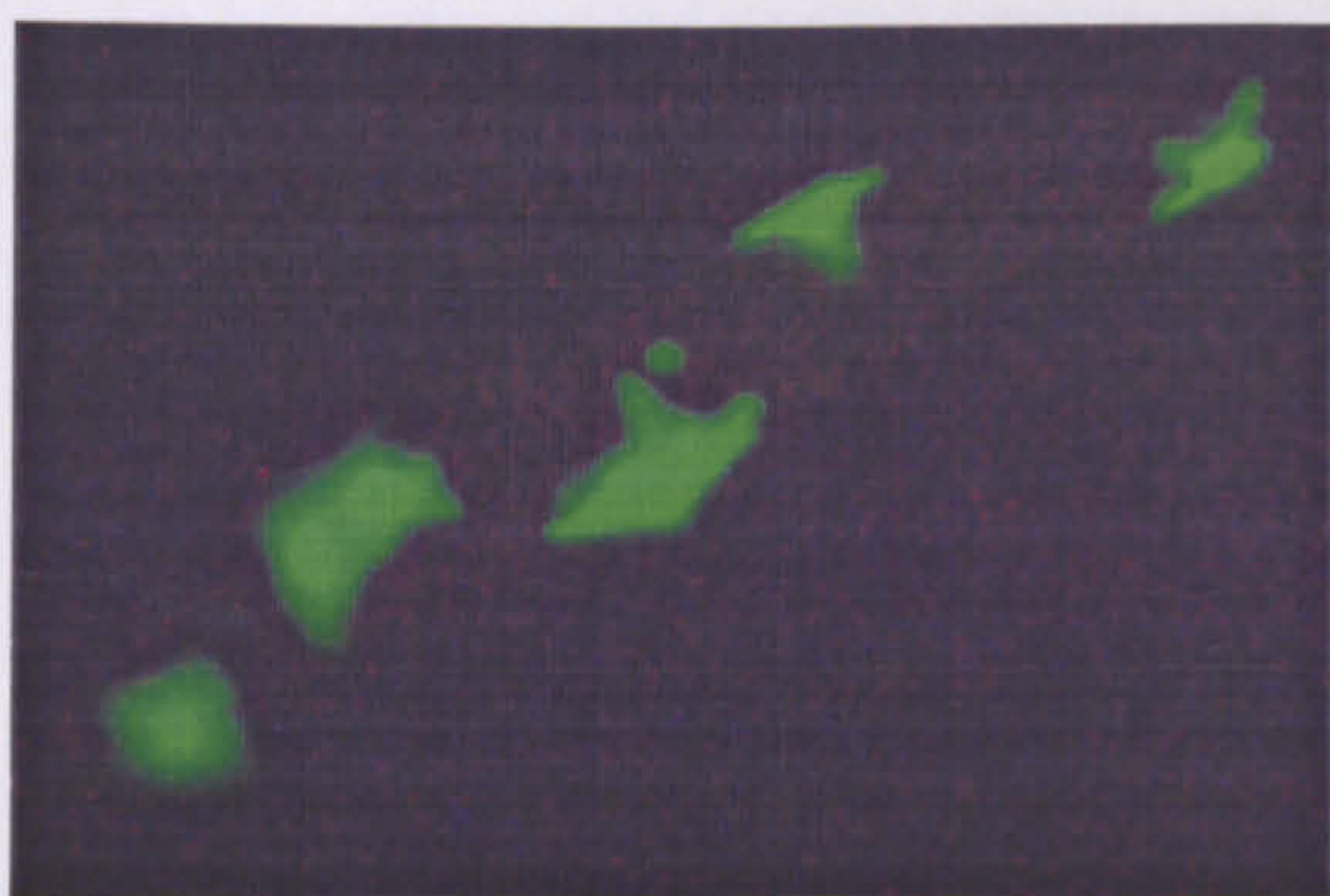
a)



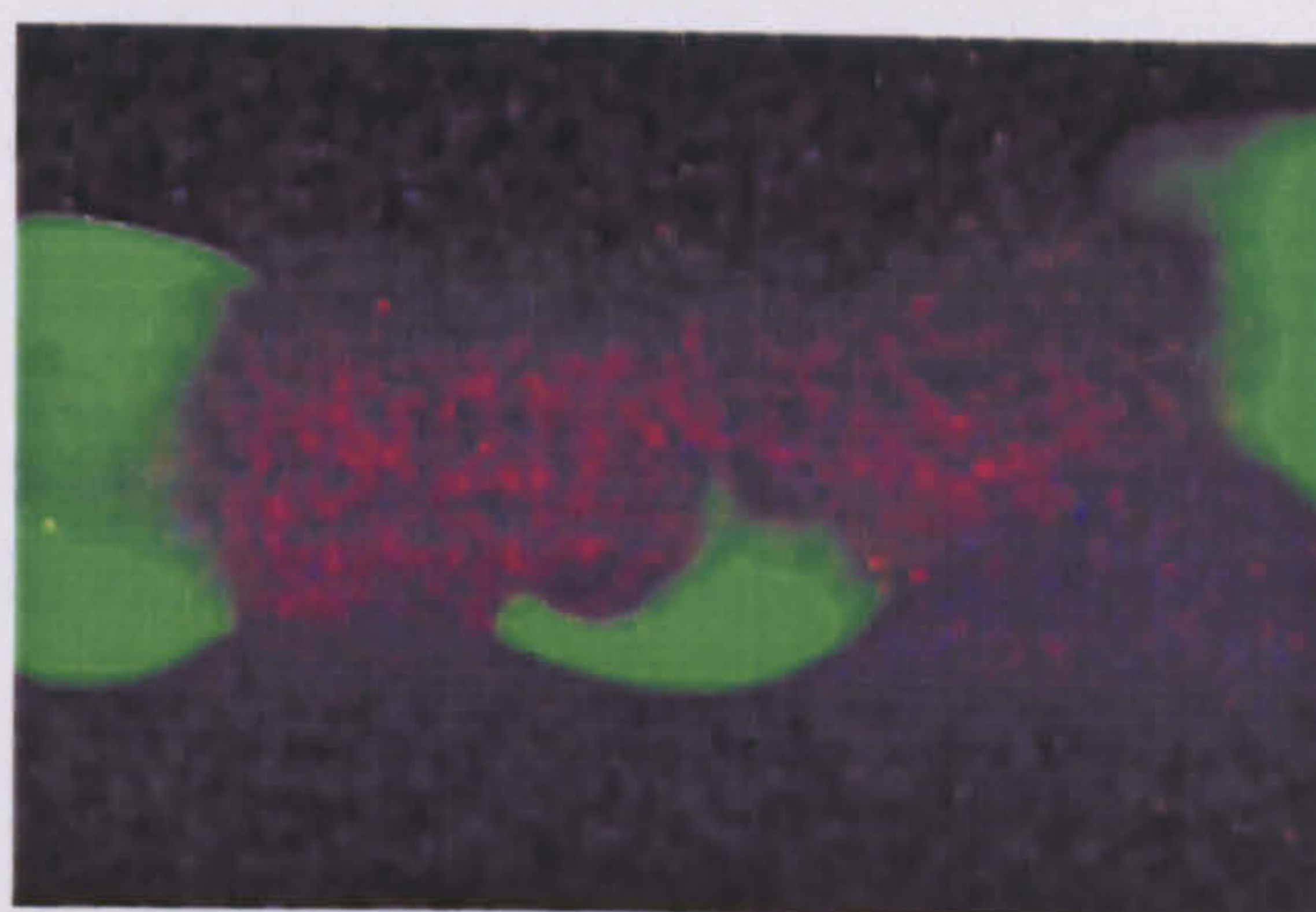
b)



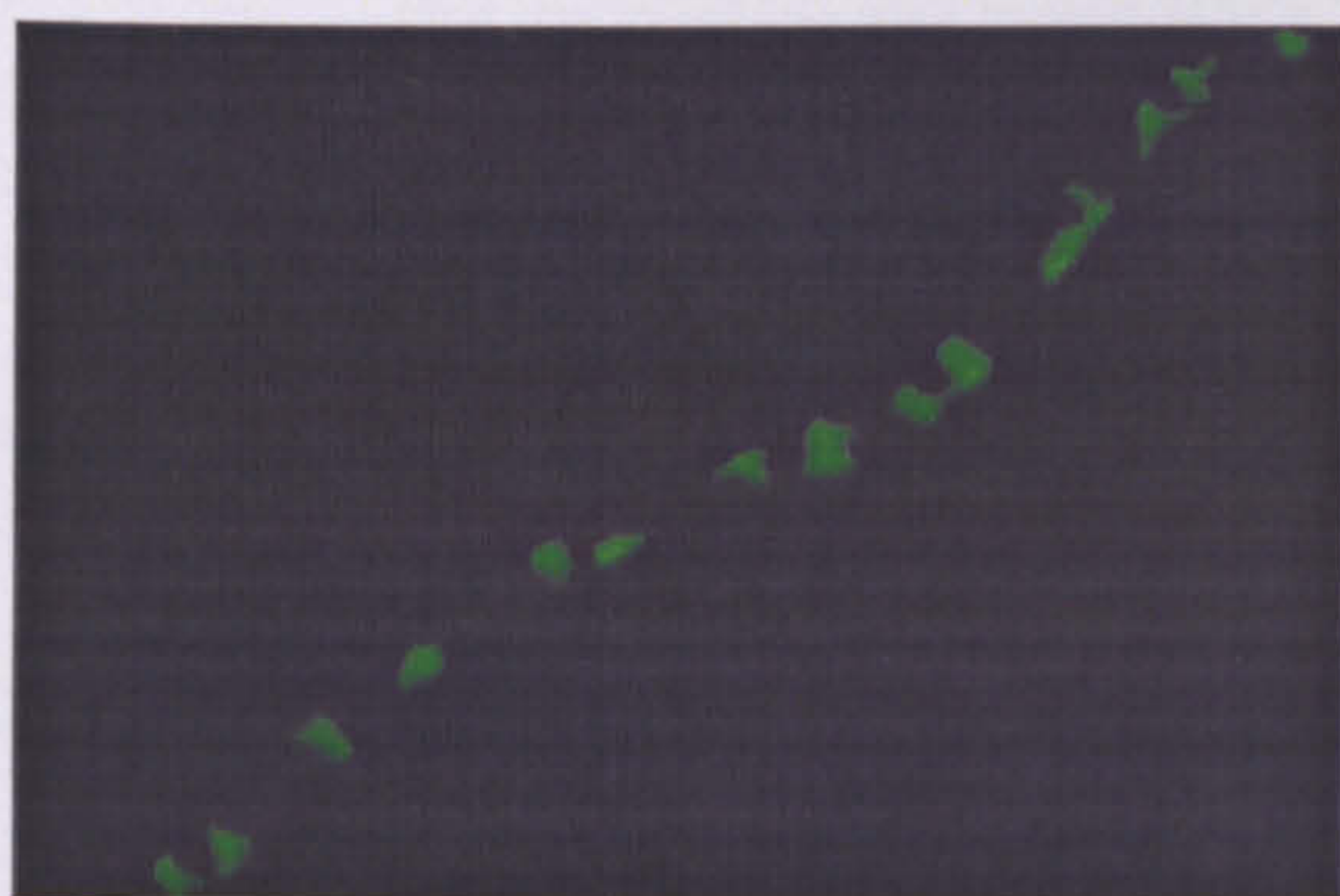
c)



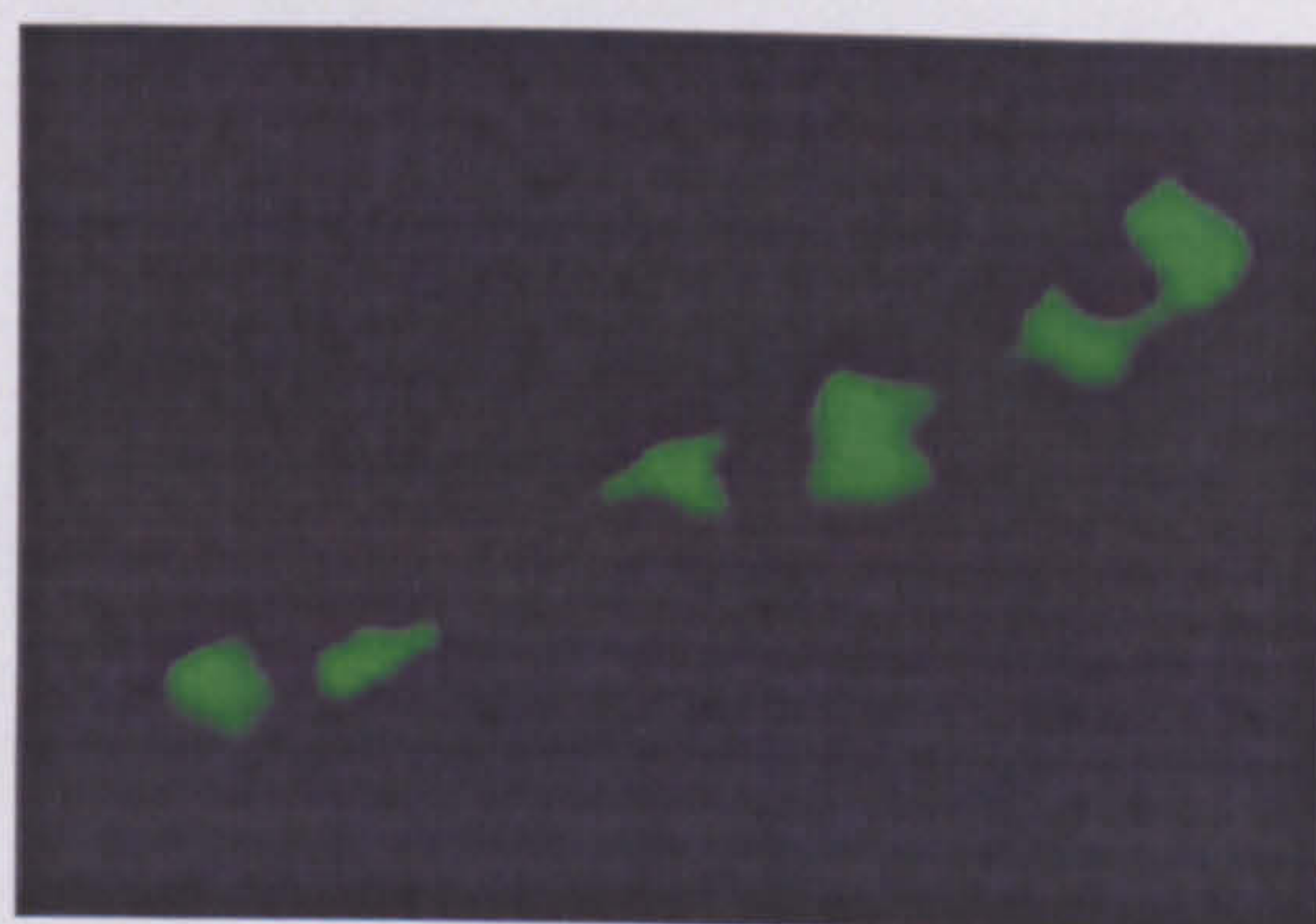
d)



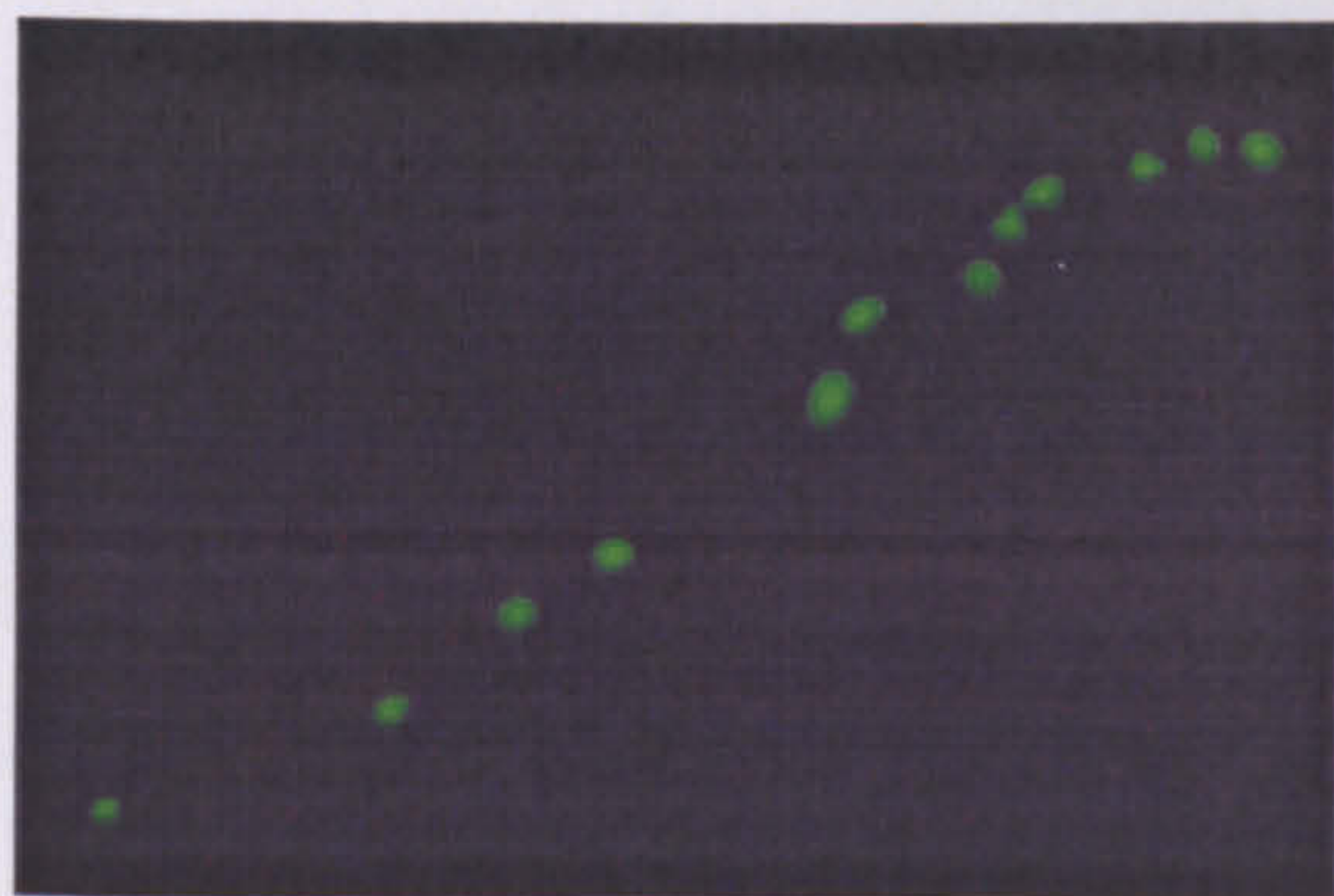
e)



f)



g)



h)

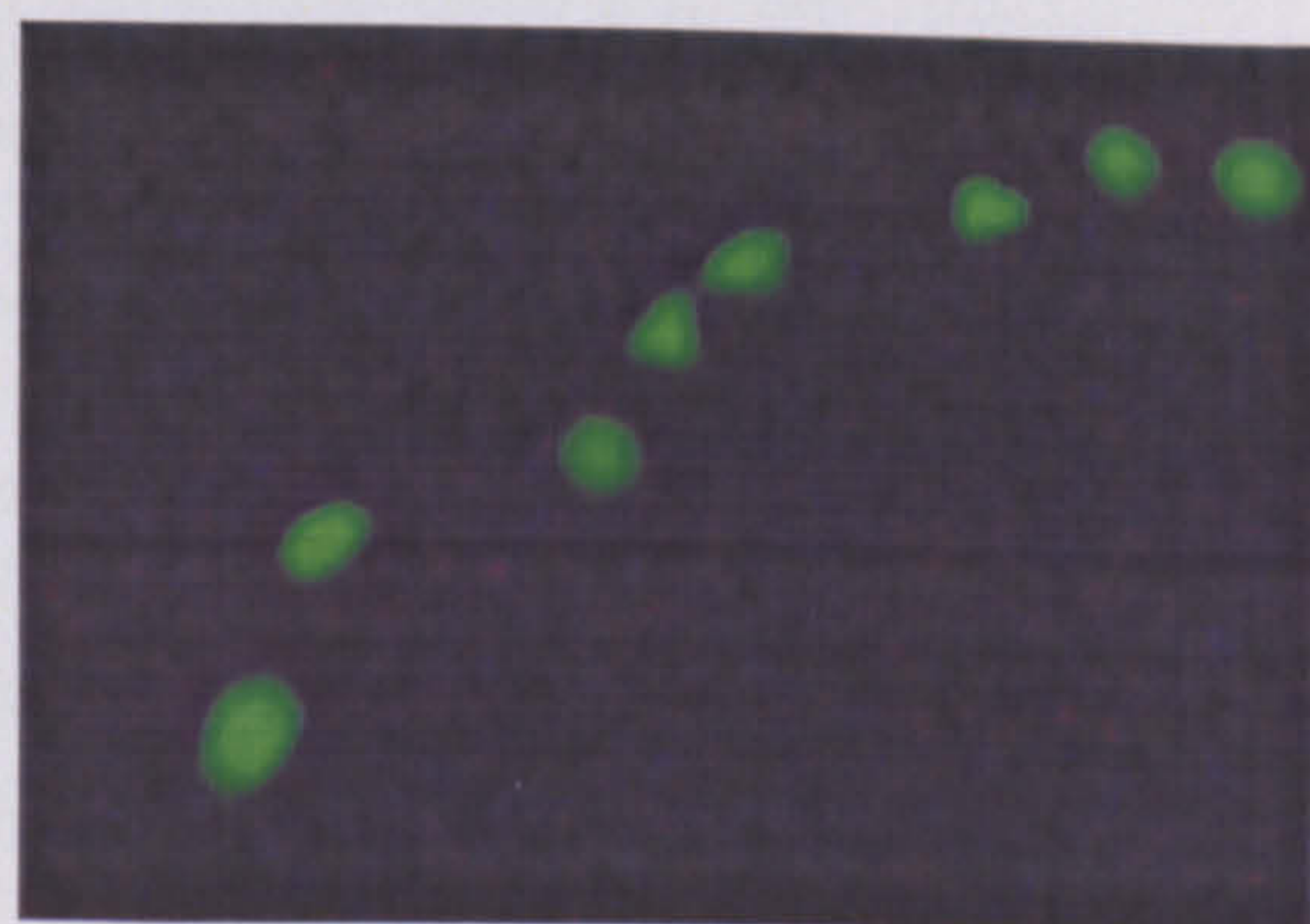


Figure 3.12. GAL4-directed GFP expression in live tubules of line C724 gives results indistinguishable from those obtained by β -gal histochemistry on fixed tissue.

- a) Stellate-shaped and bar-shaped stellate cells of anterior Malpighian tubule.
 - b) The bar-shaped ones in the distal part (initial and transitional segments).
 - c,d) The stellate-shaped stellate cells of main segment in the anterior Malpighian tubule. Red colour is due to the artifact of color adjustment on computer.
 - e) The stellate-shaped stellate cells of posterior Malpighian tubule. No bar-shaped stellate cells were observed in posterior Malpighian tubule.
 - f) The same cells at higher magnification.
 - g) The stellate cells of posterior tubule may not seem stellate-shaped all the time possibly due to the osmoregulatory status of the insect at the time of dissection.
 - h) Similar stellate cells of posterior Malpighian tubule which *are* stellate, at higher magnification.
-

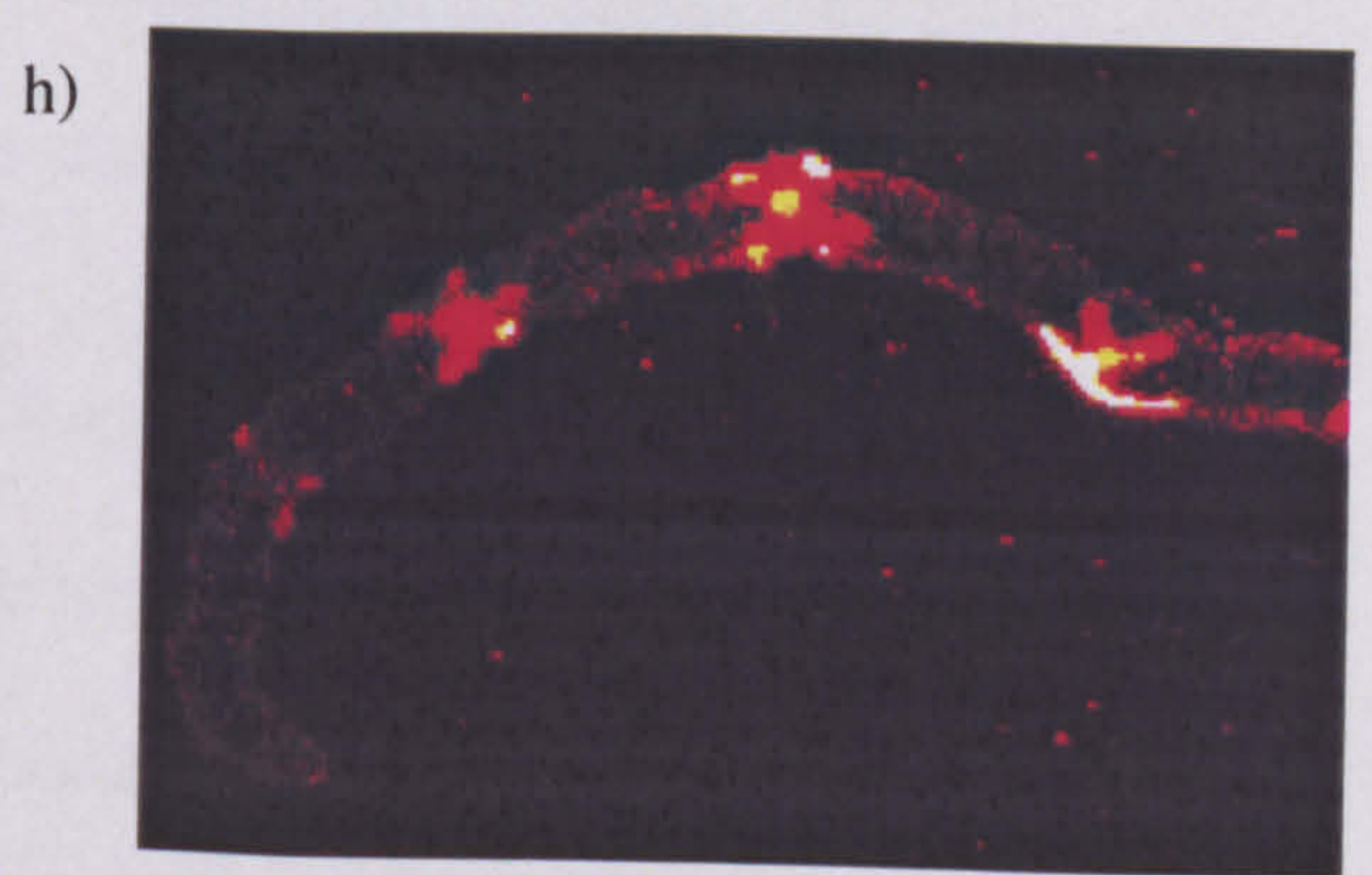
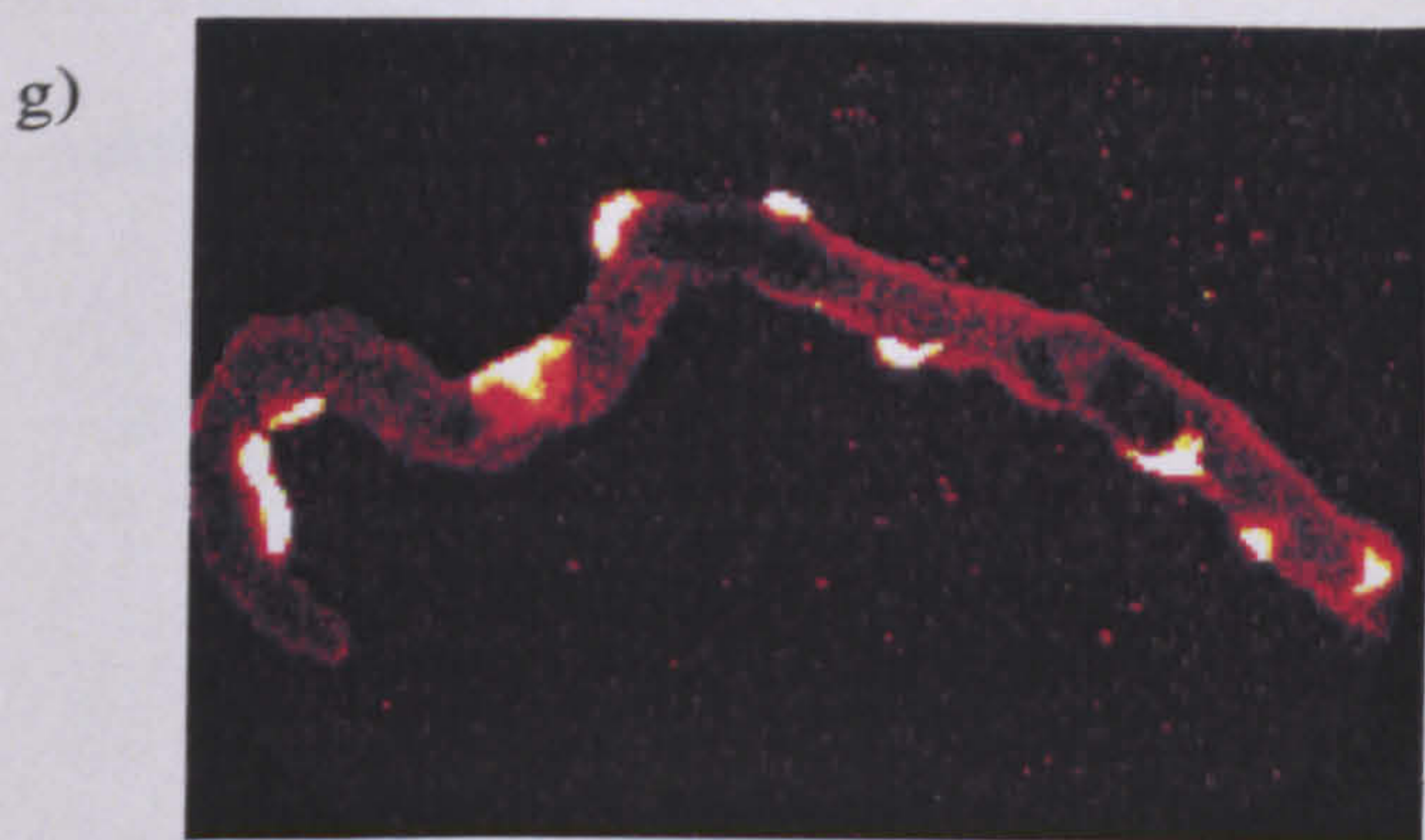
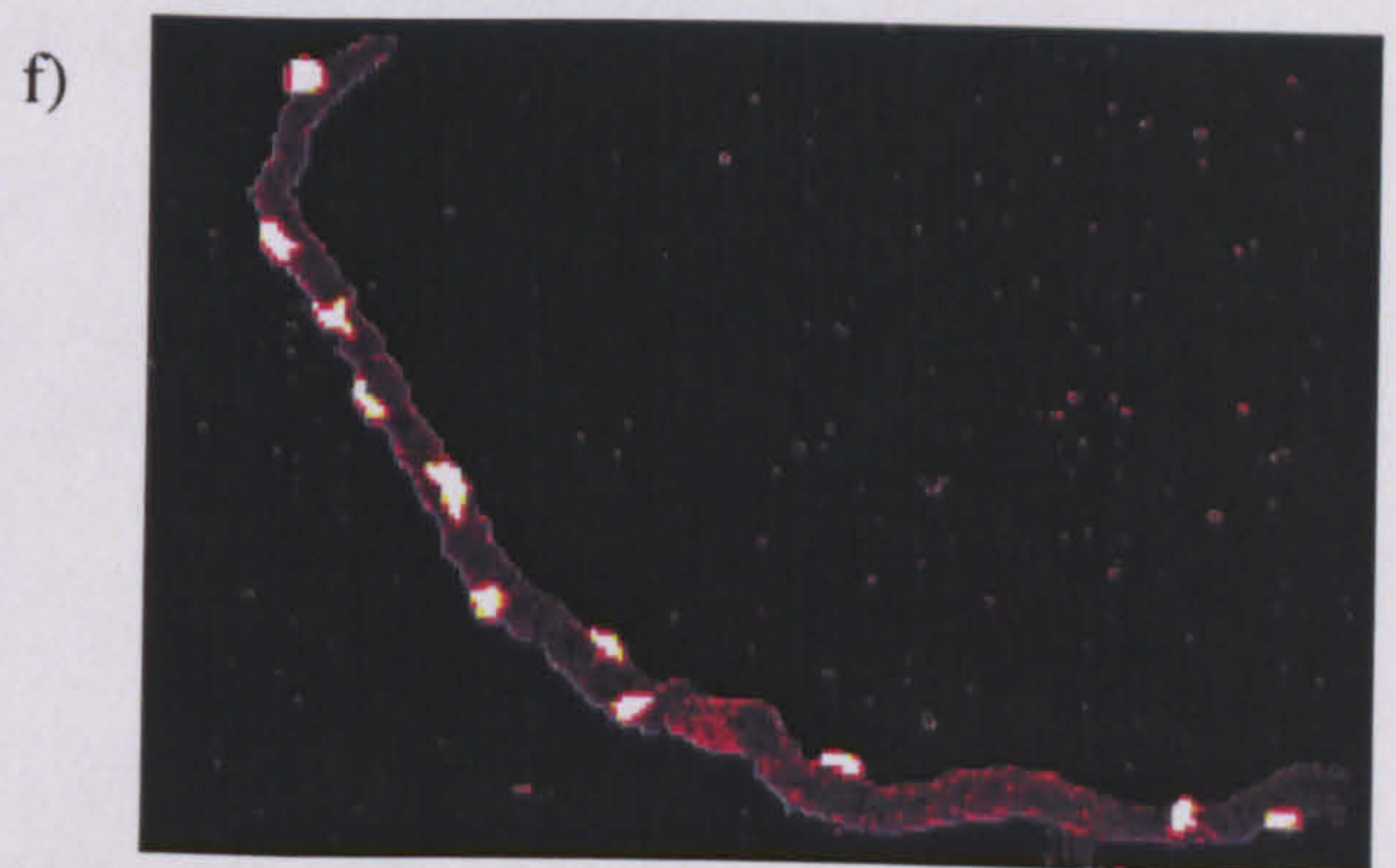
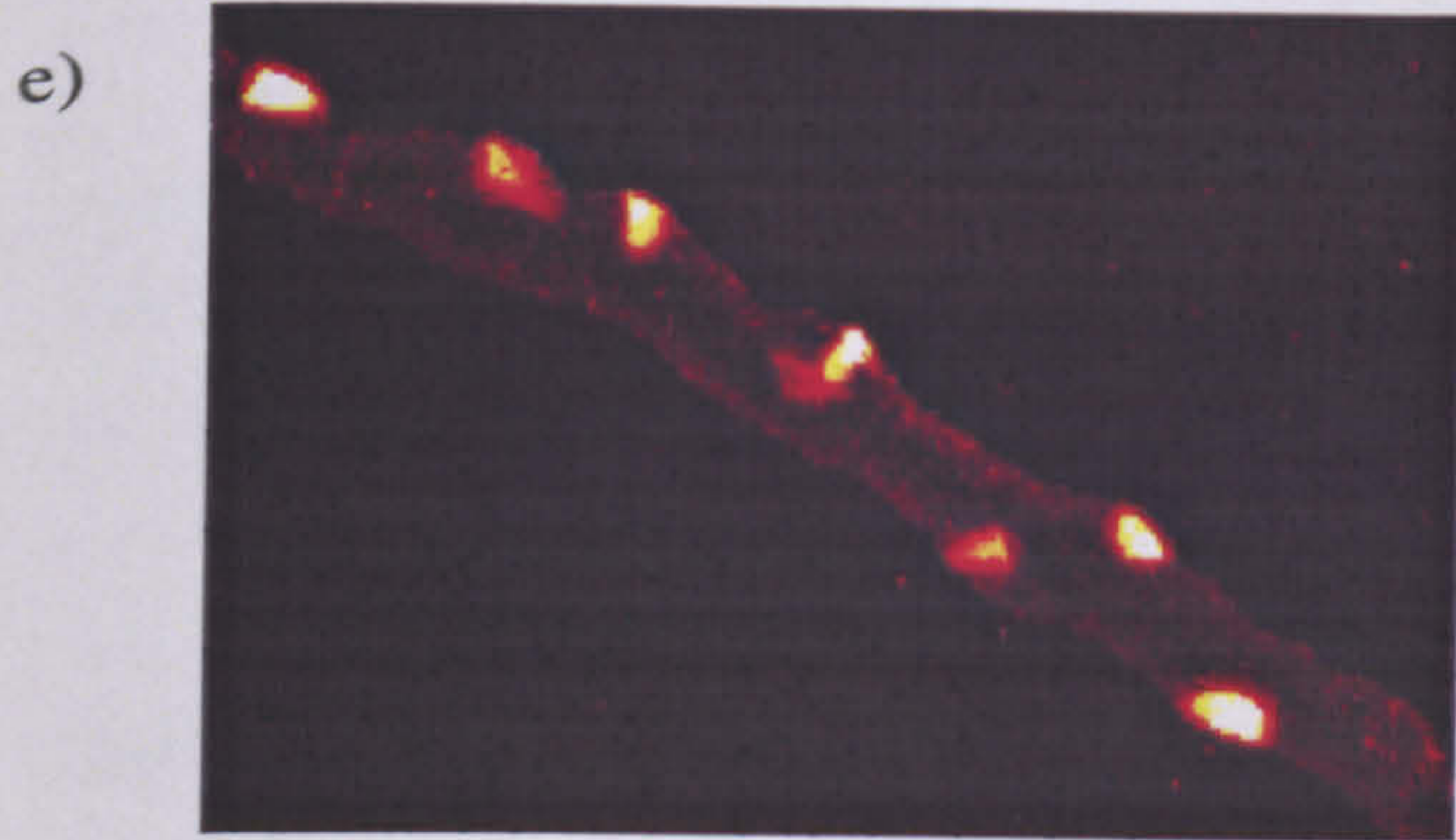
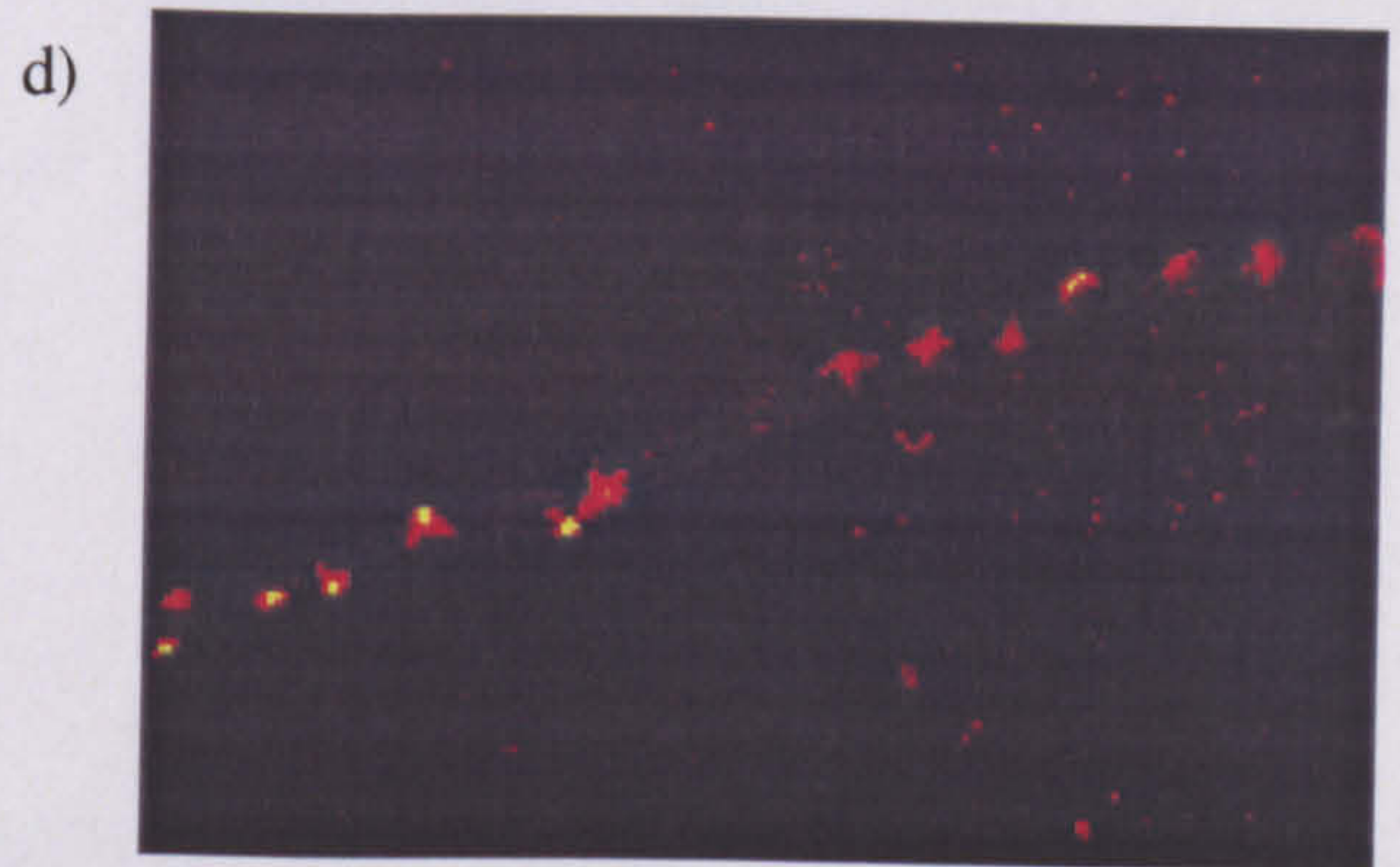
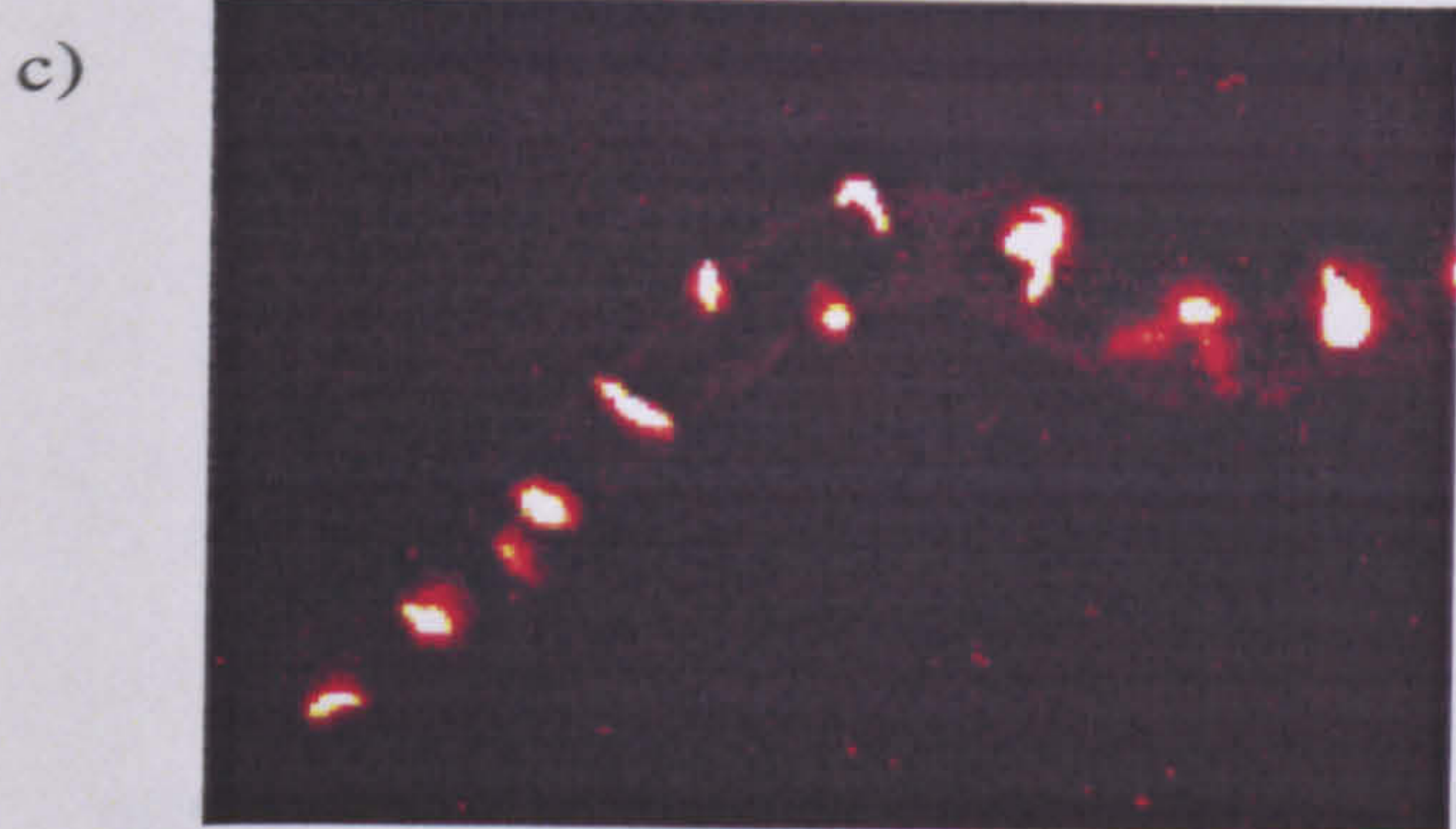


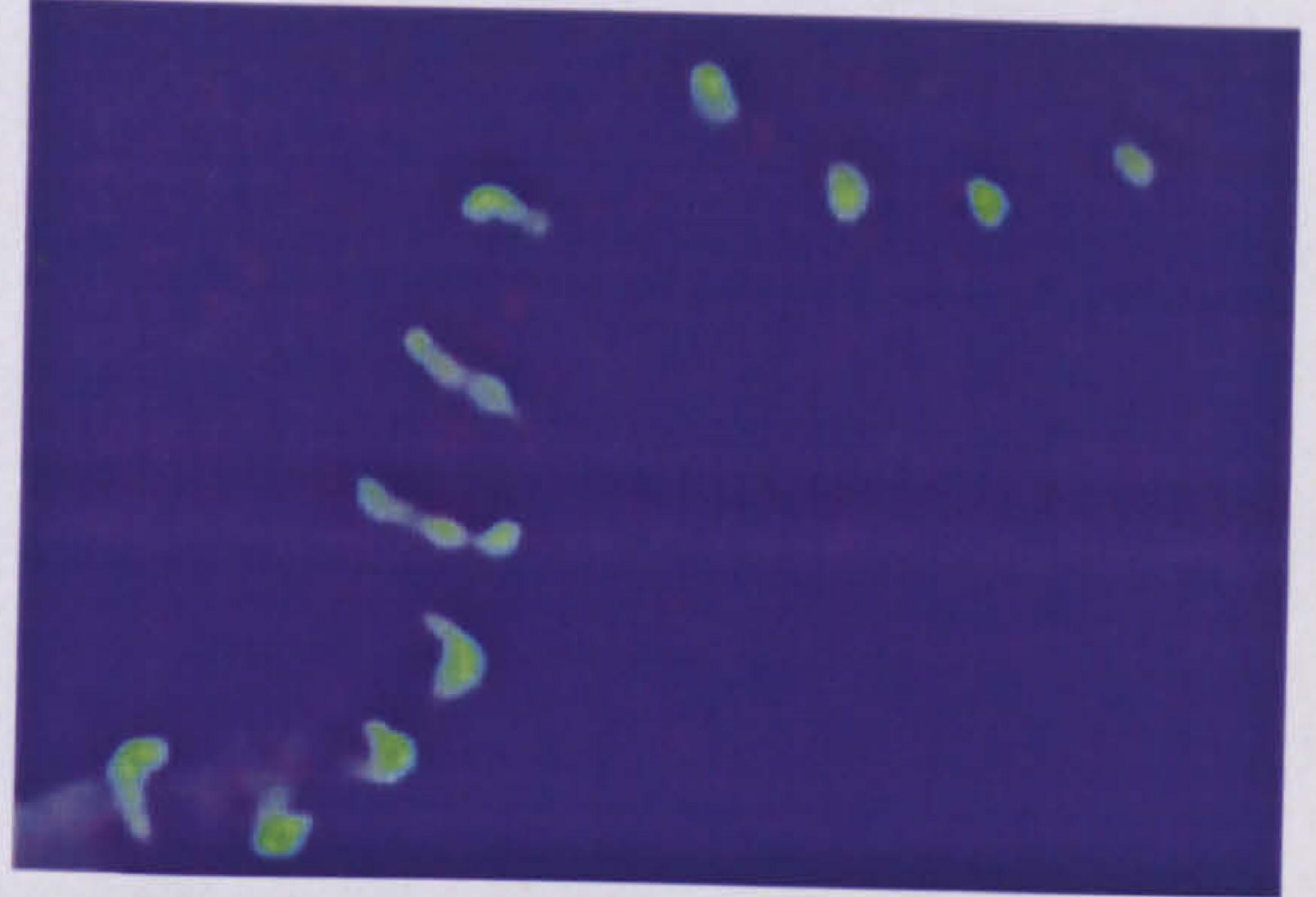
Figure 3.14. Line c710. Adult GAL4-directed GFP expression patterns of Malpighian tubules by epifluorescence.

- a) GAL4 expression can be detected in all stellate cells both stellate-shaped cells of main segment and bar-shaped ones of initial and transitional segment in the anterior Malpighian tubules.
 - b) The higher magnification of bar-shaped cells.
 - c) The cross-shaped stellate cells of the main segment of the anterior Malpighian tubules at higher magnification.
 - d) A single cell from the main segment of the anterior Malpighian tubules at higher magnification.
 - e) The stellate cells of the posterior Malpighian tubules with overall view.
 - f) Some of the stellate cells of the posterior Malpighian tubules
 - g) Some (cross-shaped) stellate cells of posterior Malpighian tubules .
 - h) A single stellate cell from the main segment of the posterior Malpighian tubules at higher magnification.
-

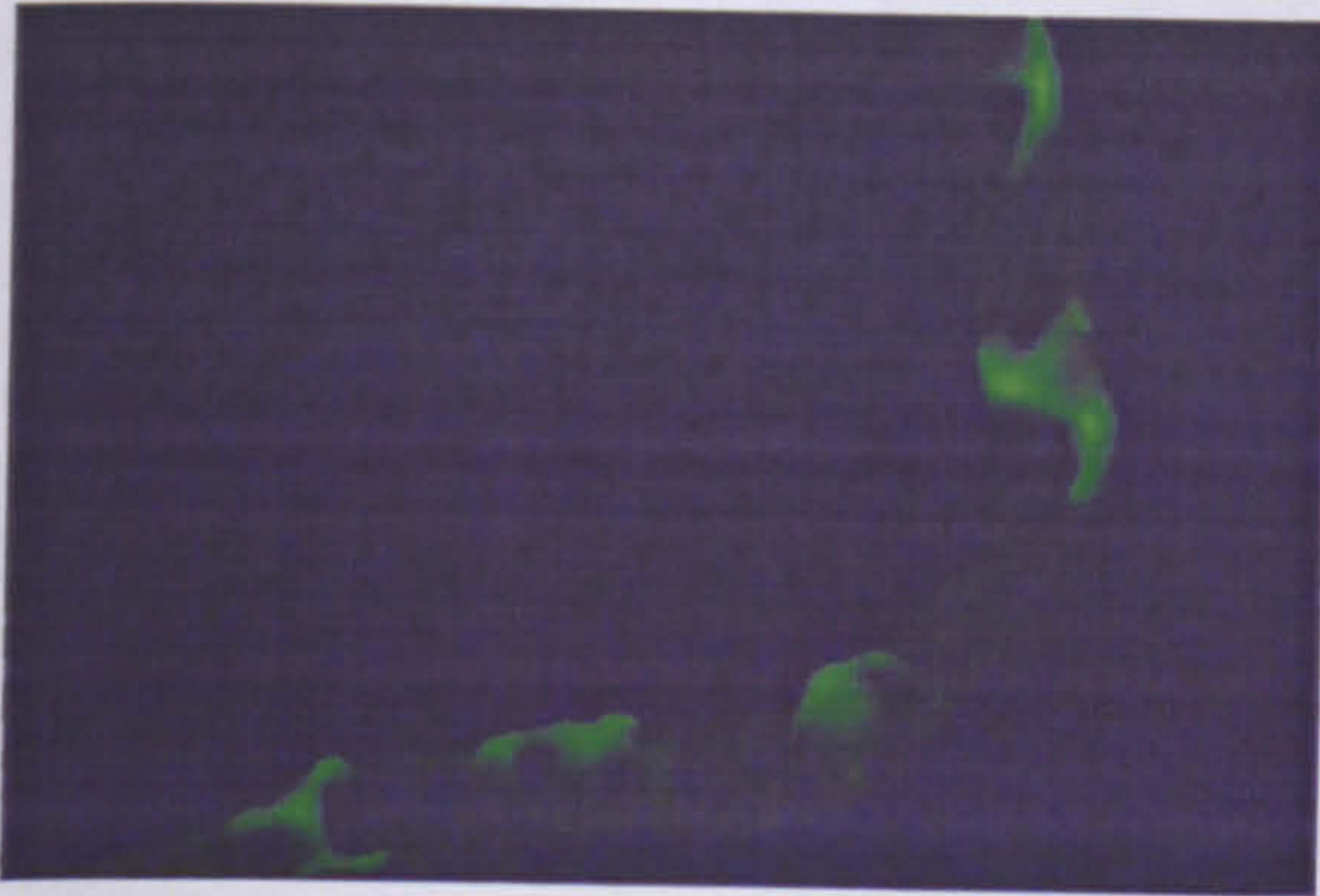
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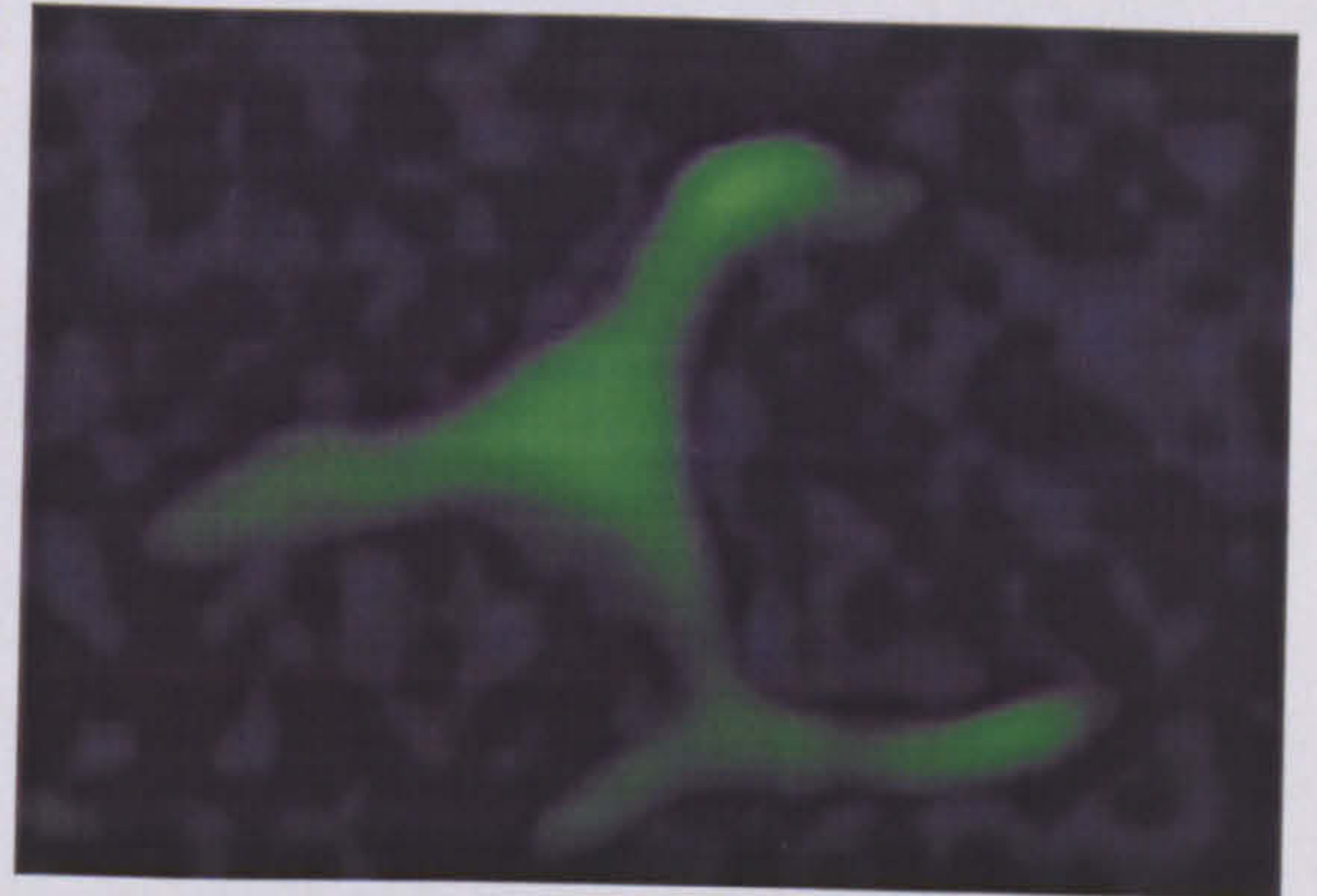
b)



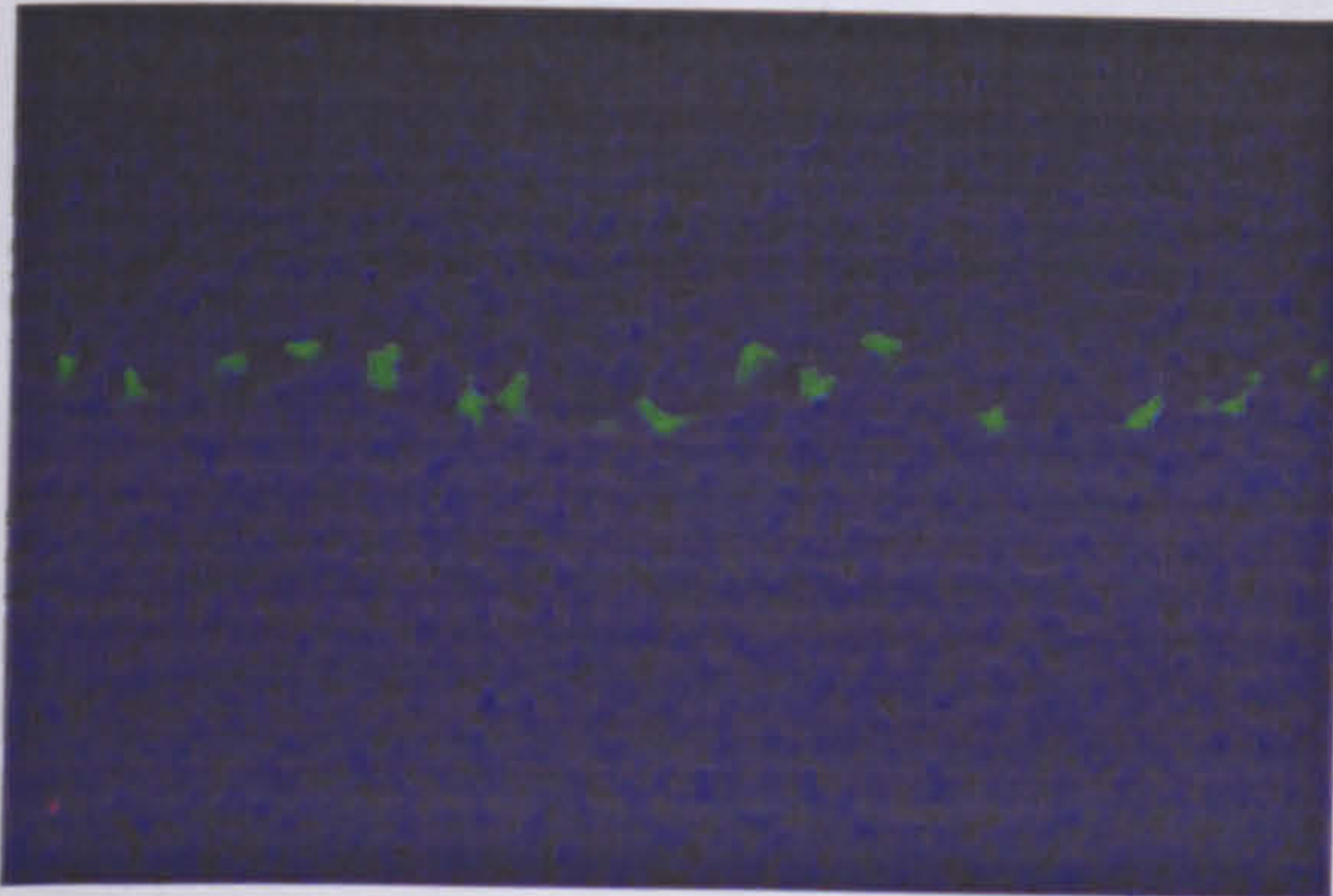
c)



d)



e)



f)

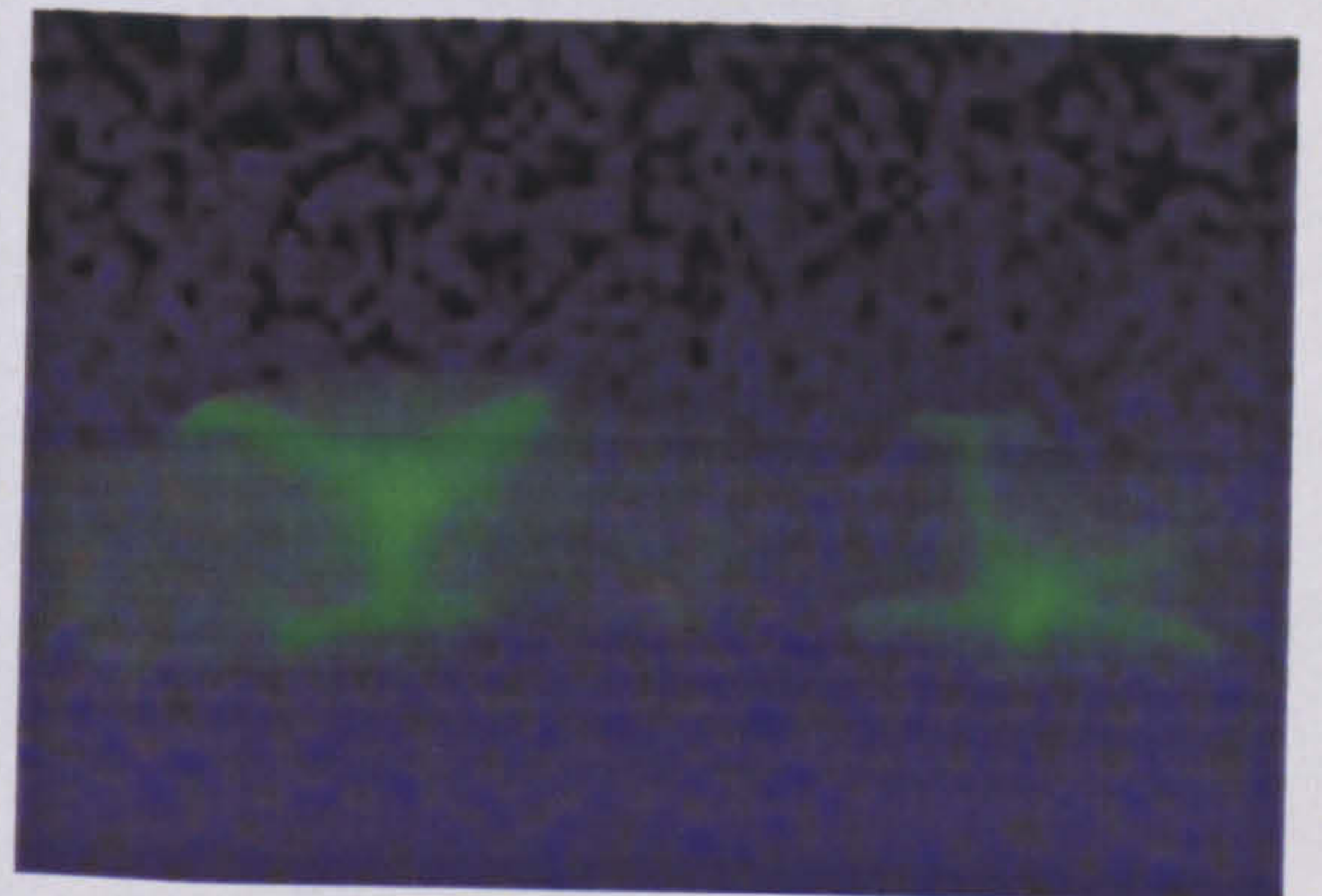
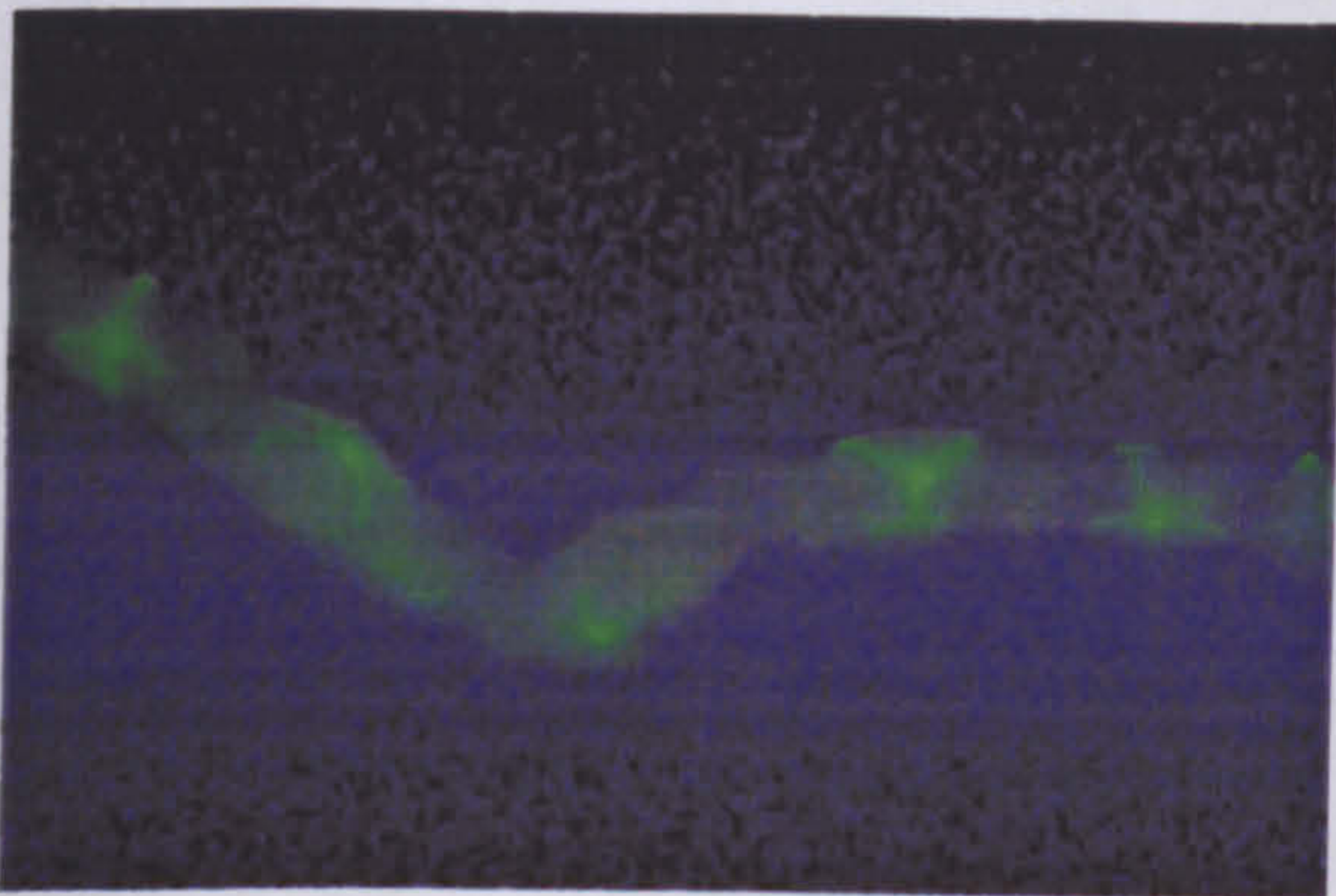
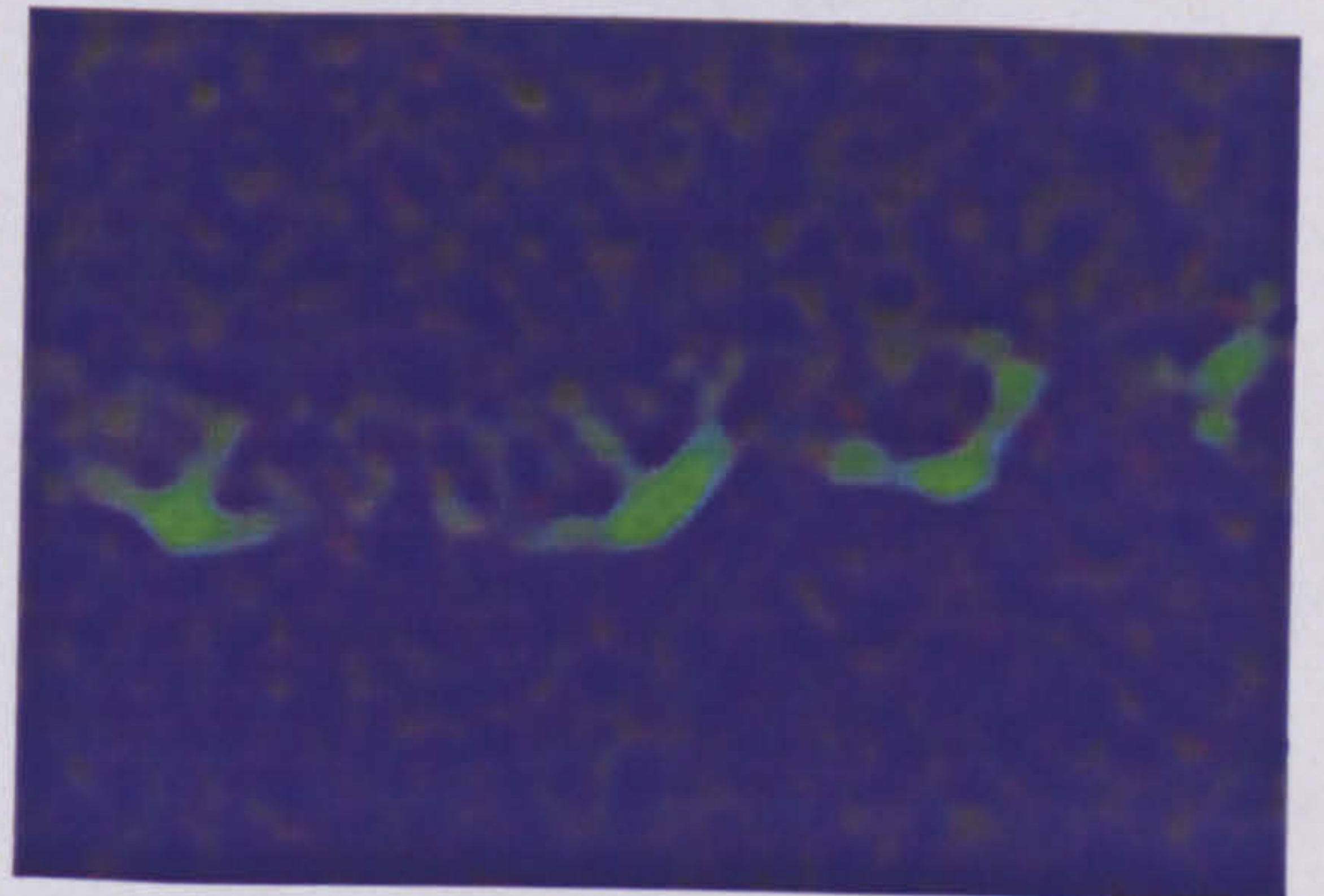


Figure 3.15. Line c649. GAL4-directed GFP expression patterns of adult Malpighian tubules.

a,b) This line marks only the bar-shaped stellate cells of initial and transitional segment of anterior Malpighian tubules.

c,d) The bar-shaped stellate cells at higher magnification.

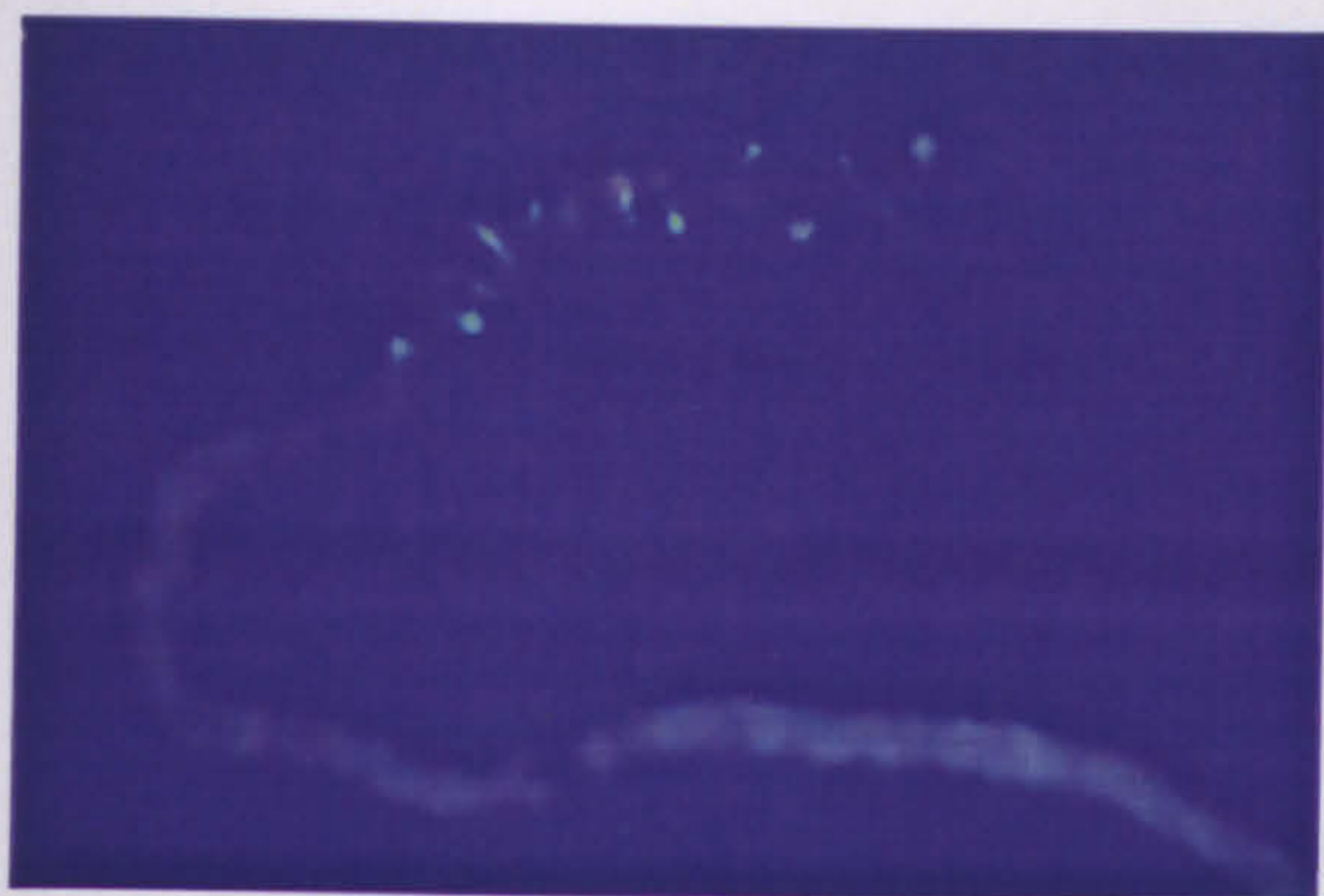
e) Three bar-shaped stellate cells at higher magnification.

f) No stellate cell expression is observed in the posterior Malpighian tubules in the same line (C649) as in the anterior tubule main segment.

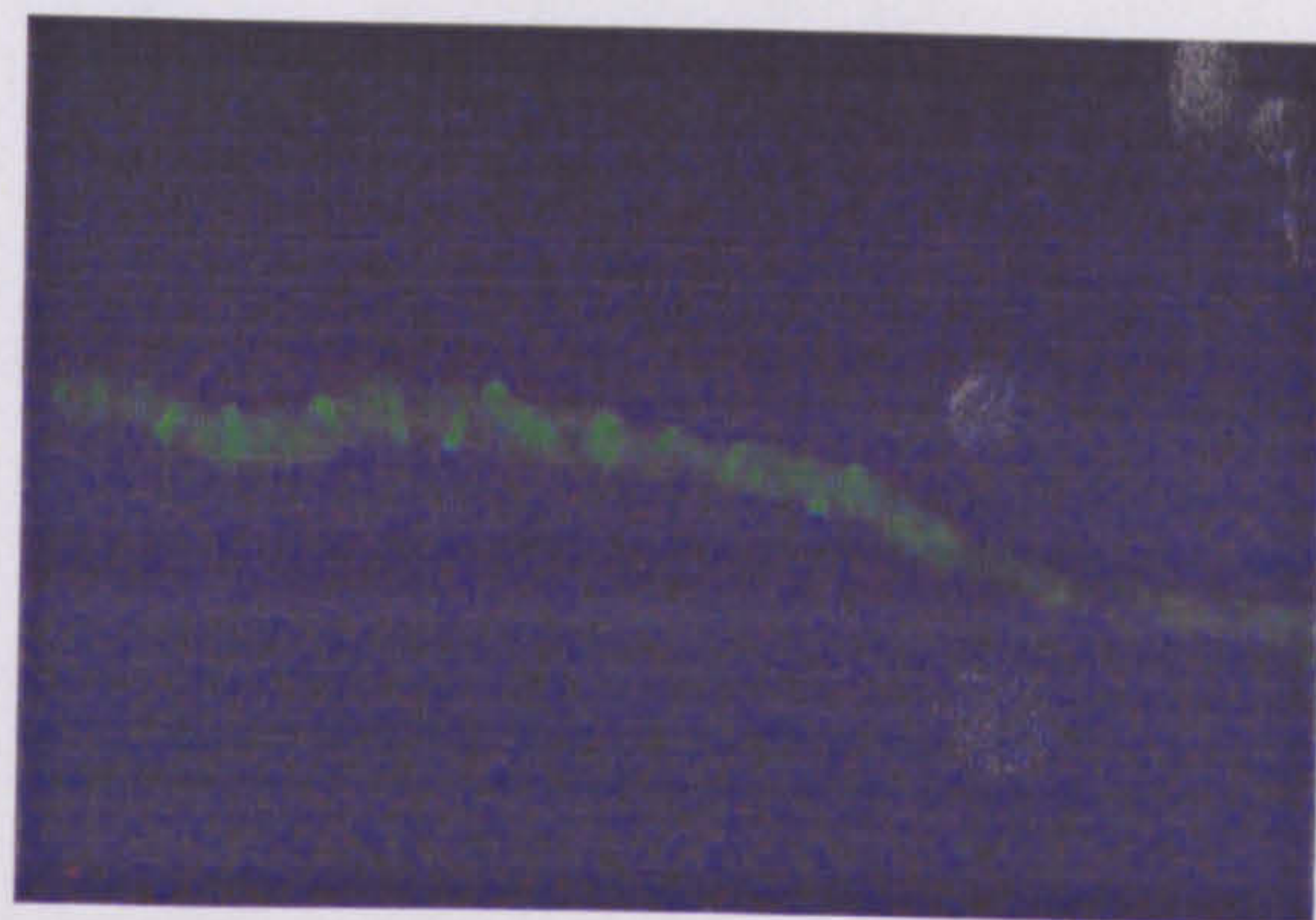
g) GAL4 is also expressed in the tiny cells of lower tubule.

h) The tiny cells at higher magnification.

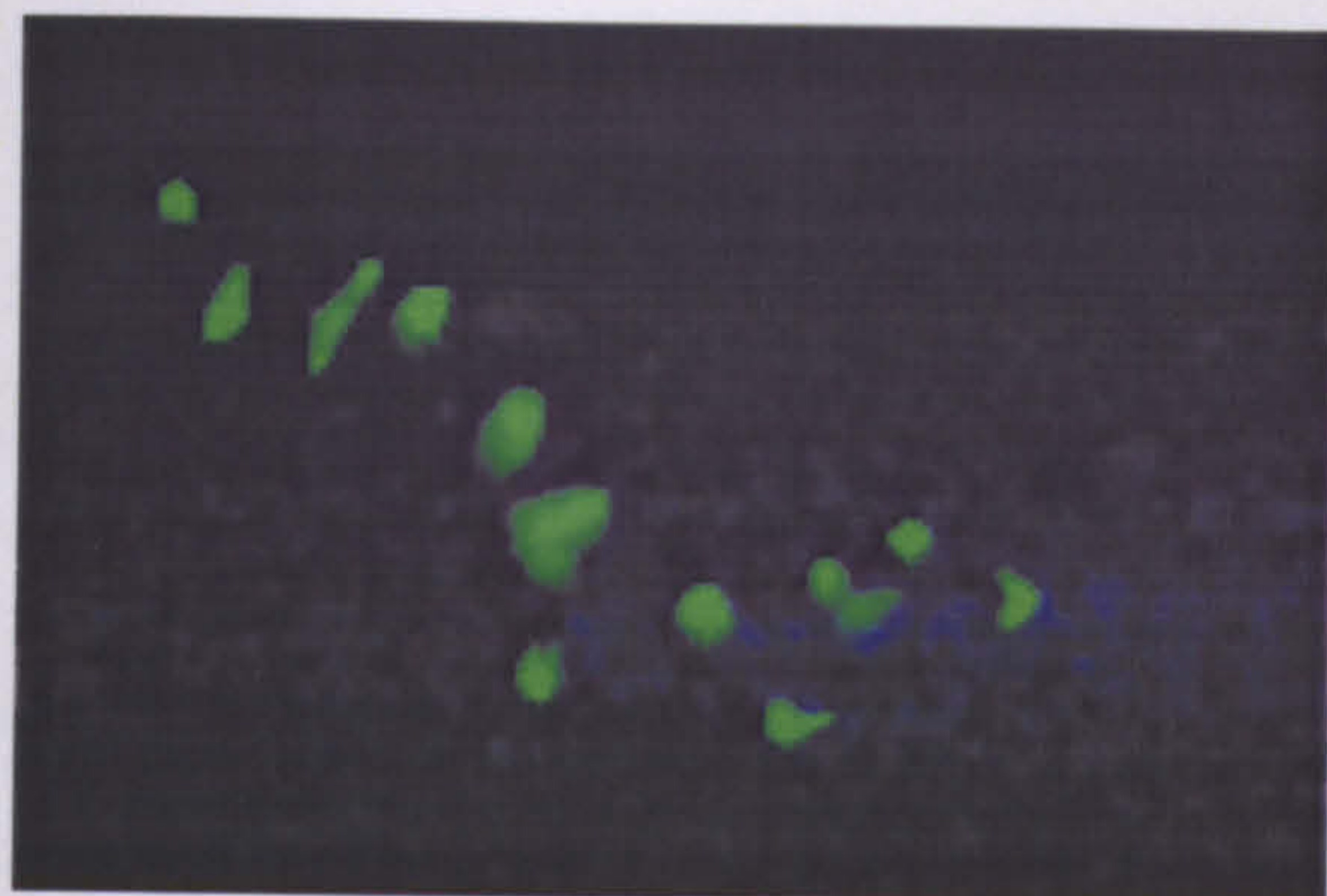
a)



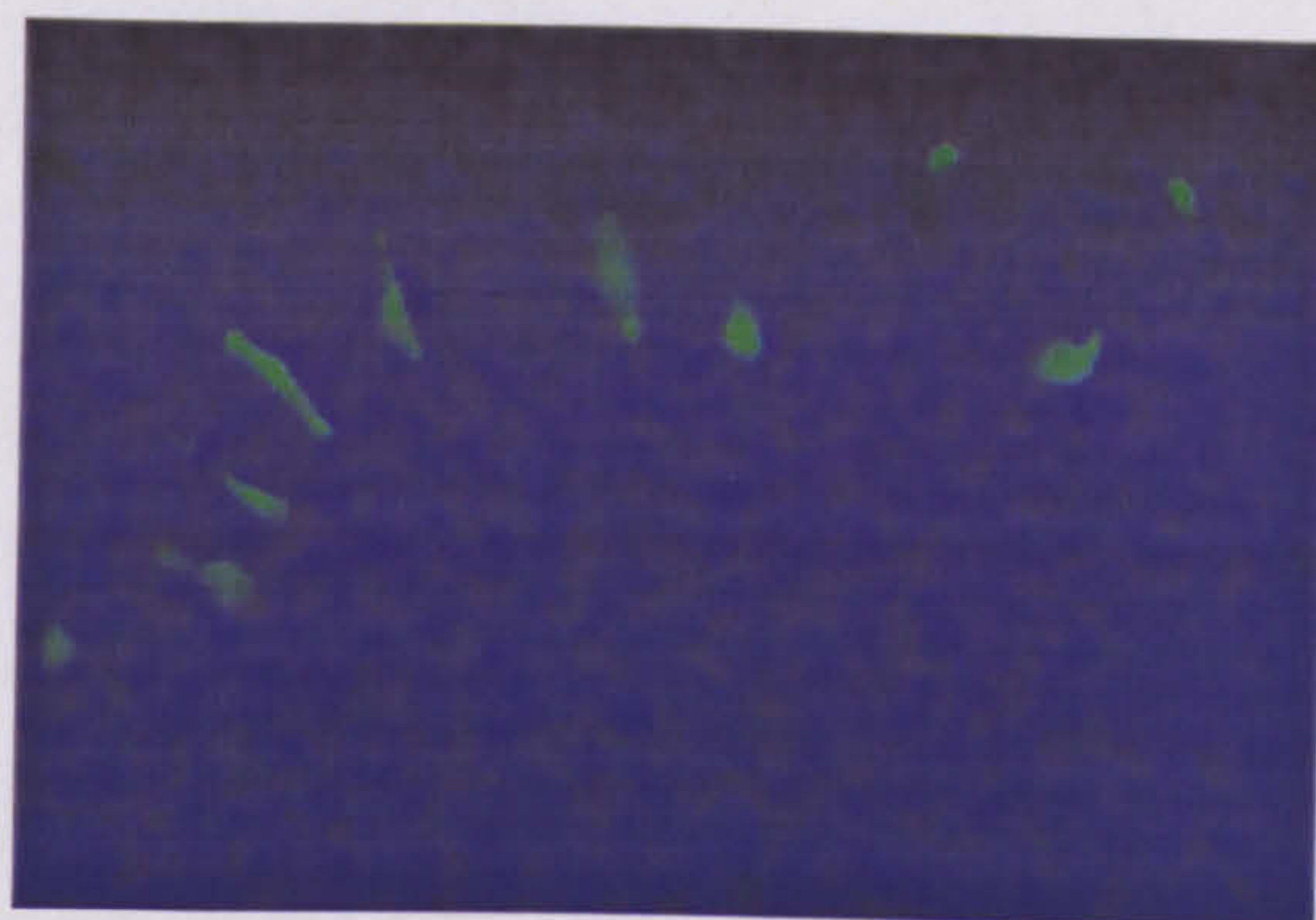
b)



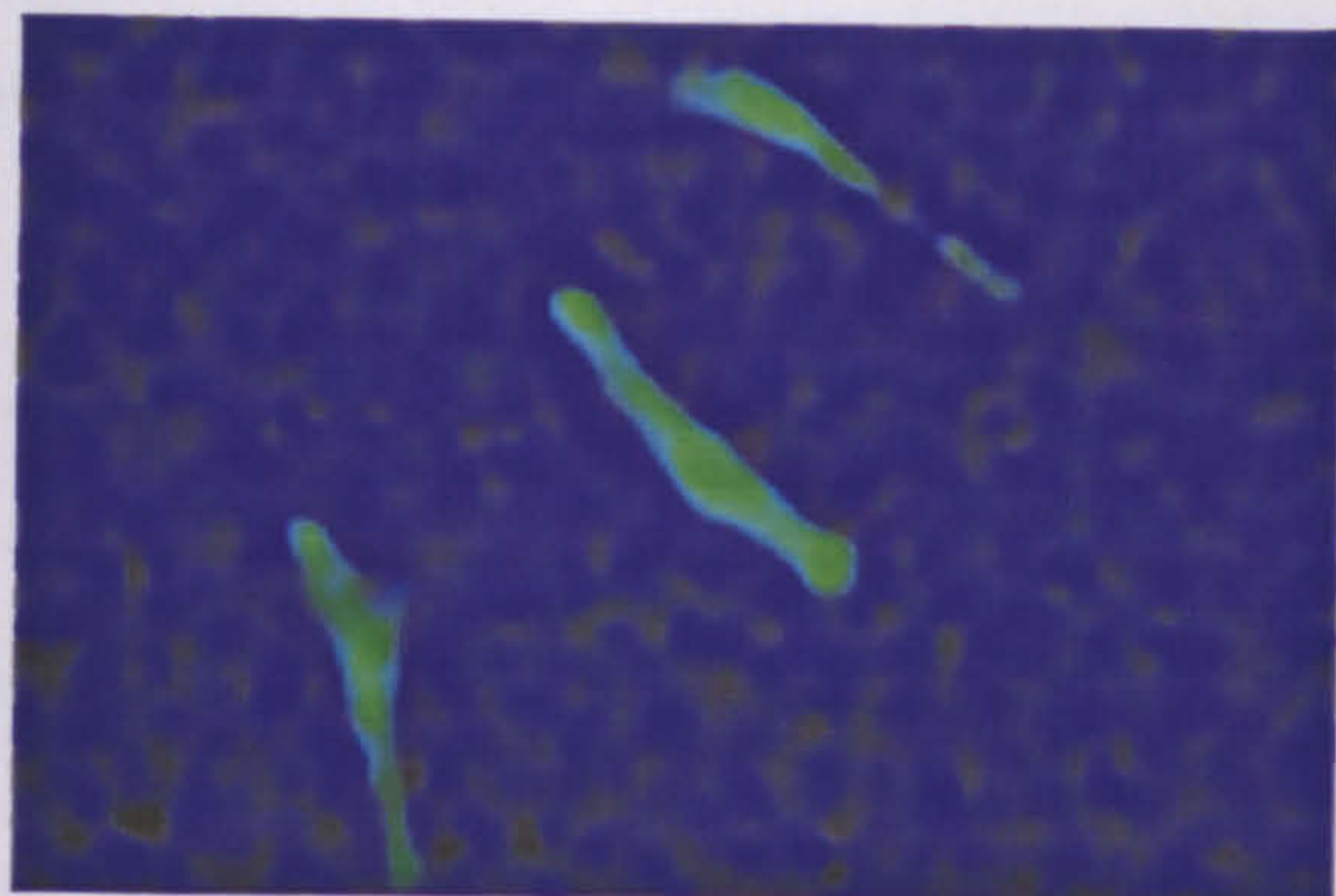
c)



d)



e)



f)

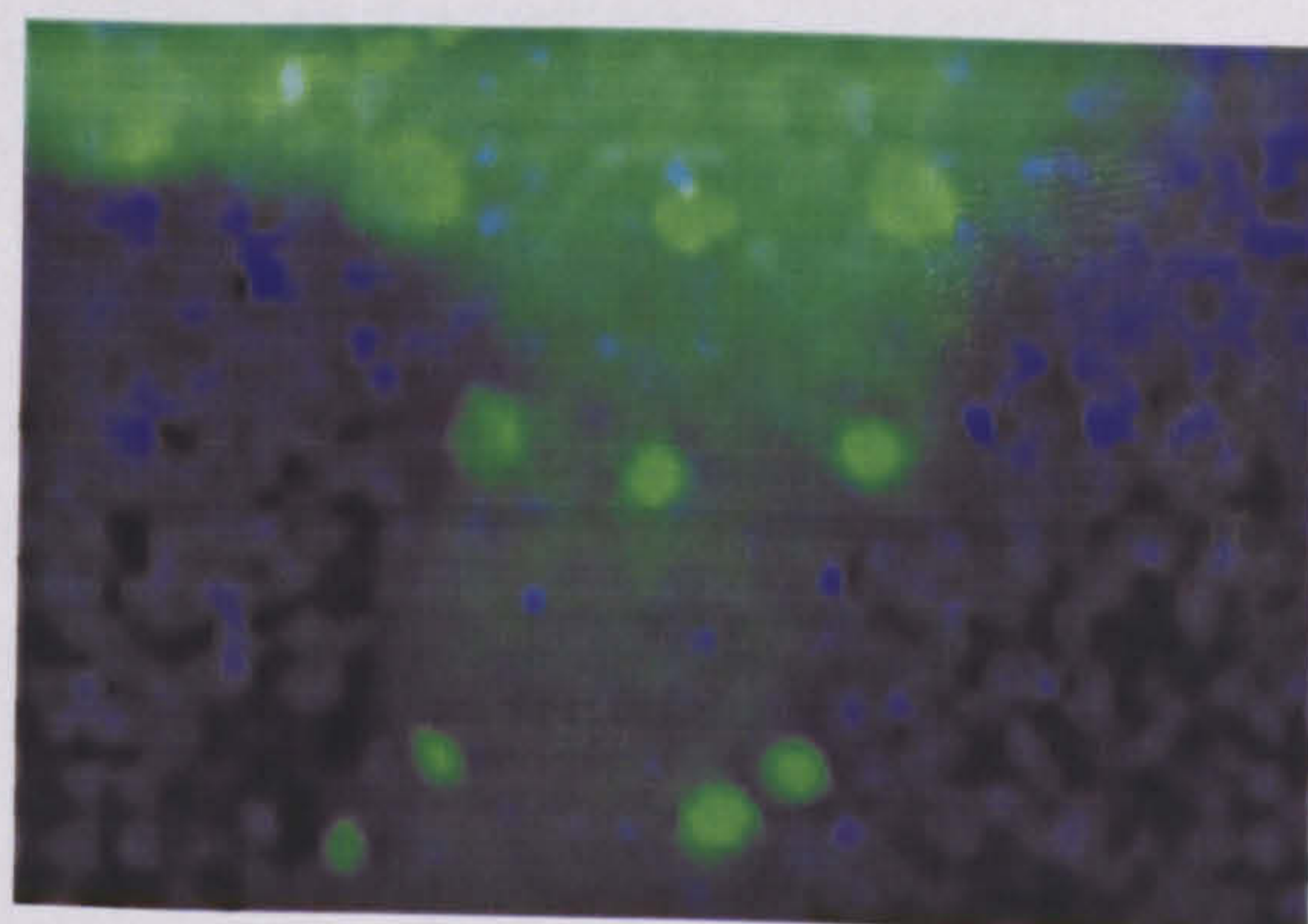
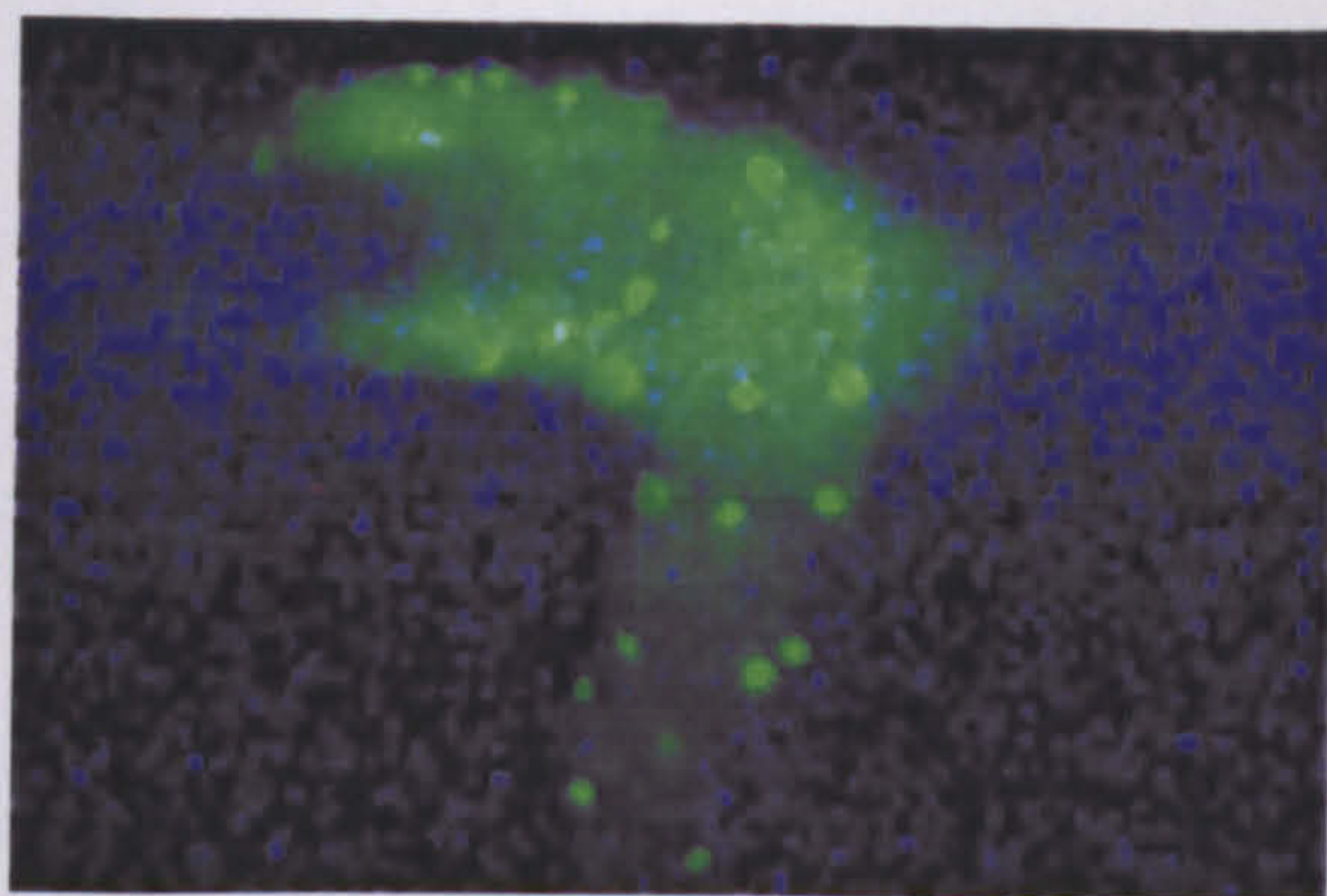
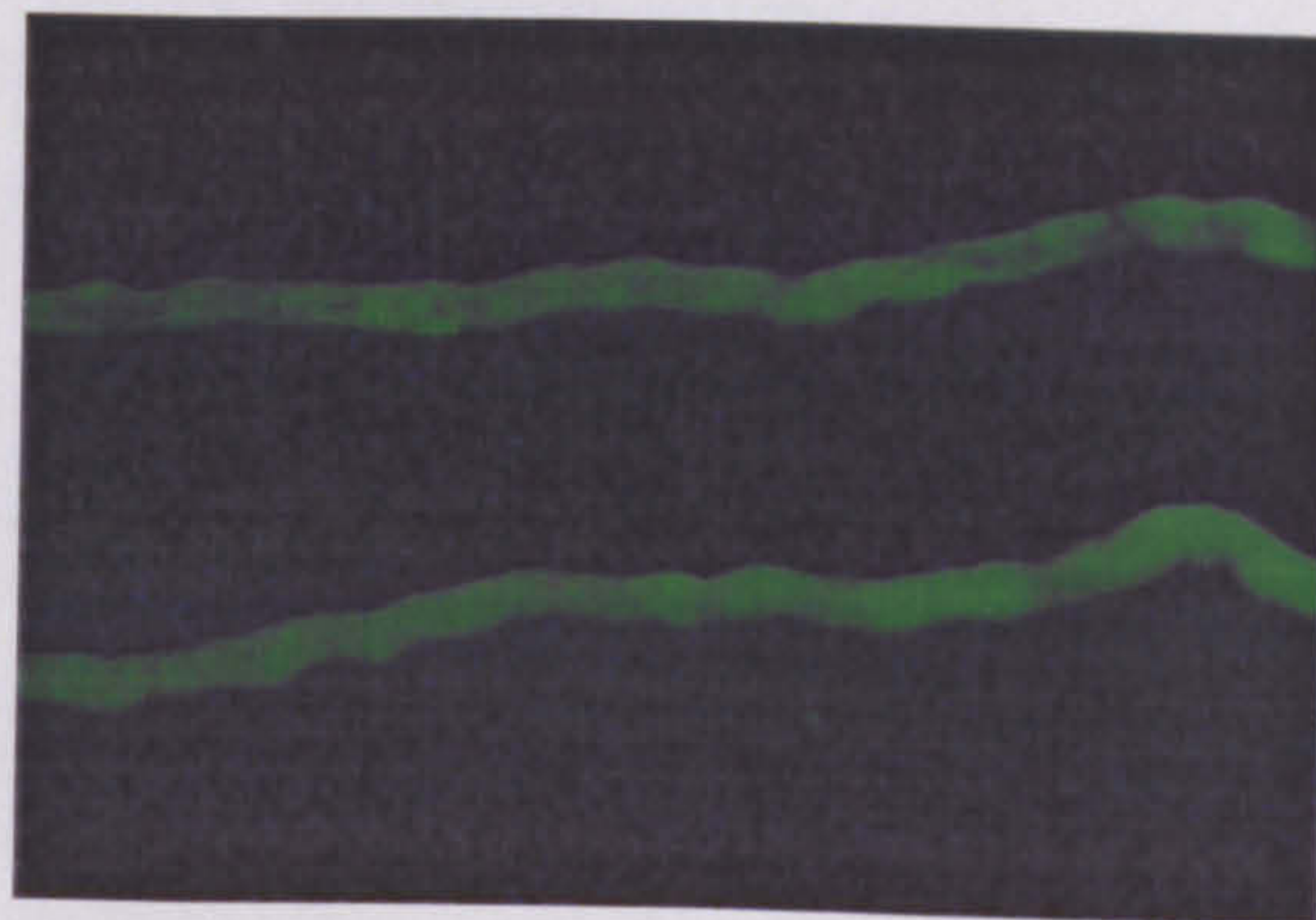
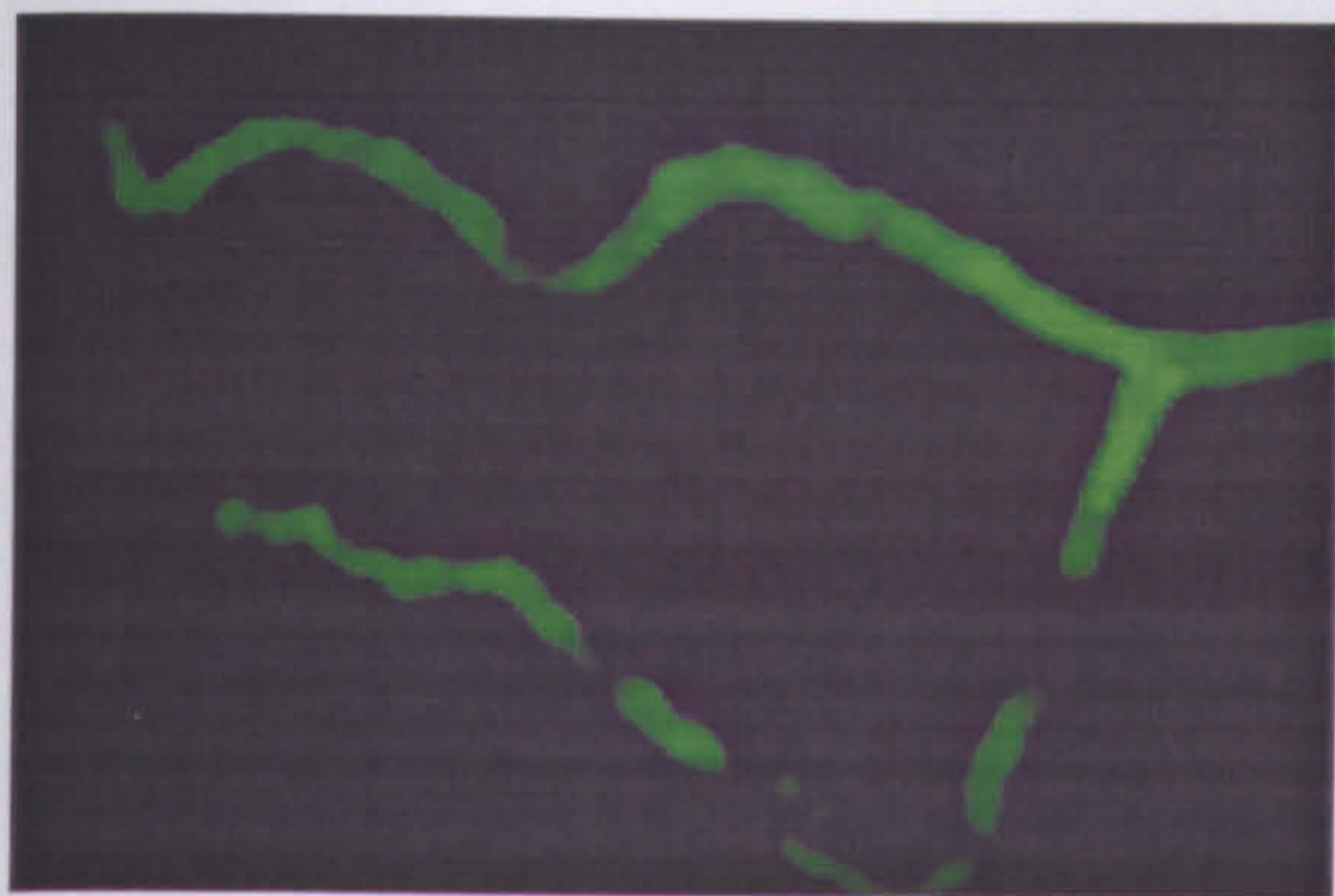


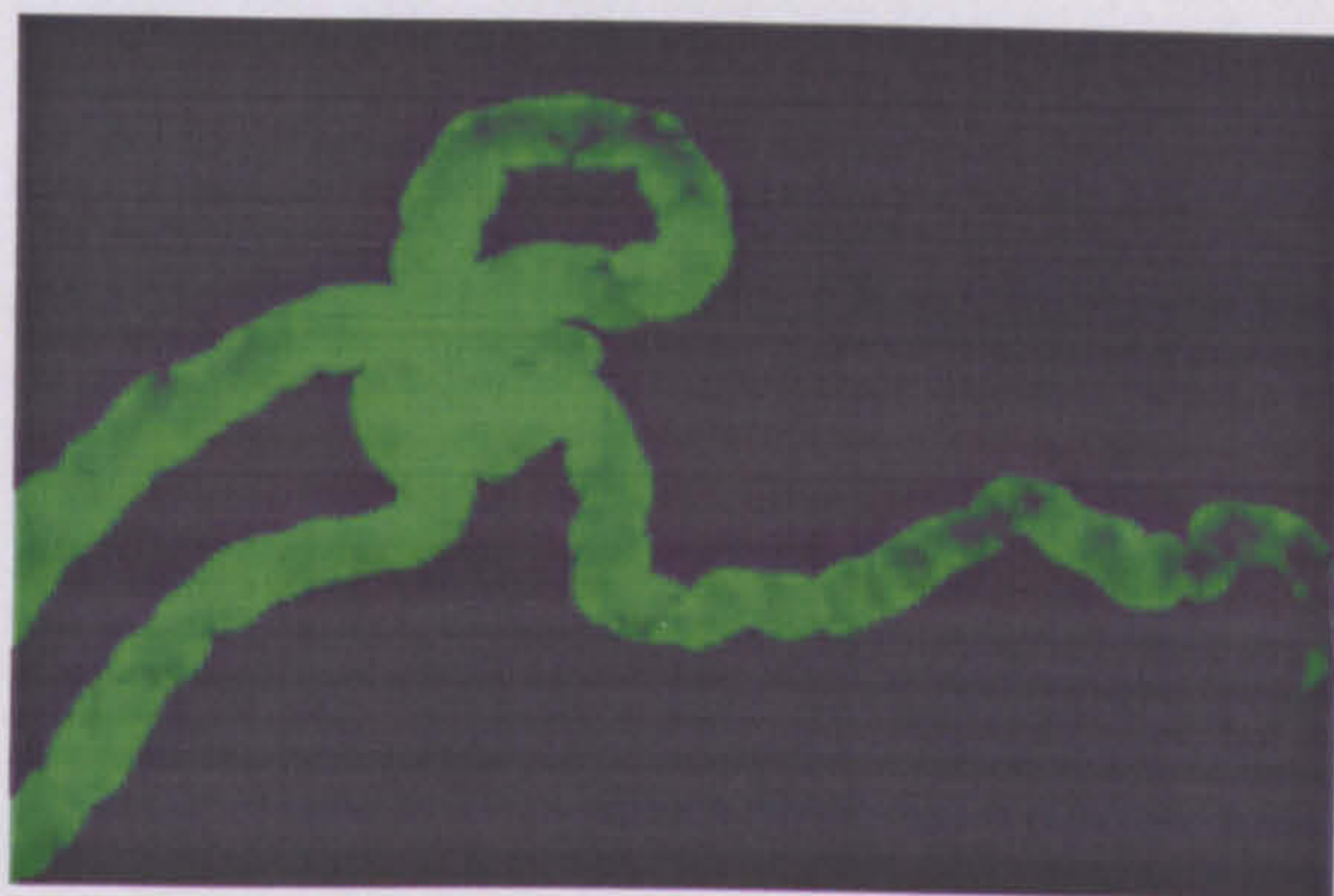
Figure 3.16. Line c42. GAL4-directed GFP expression in adult Malpighian tubules by epifluorescence.

- a) The overall view of GAL4 expression in the principal cells of tubules except for the non-transporting distal part of tubules.
 - b) The overall view of posterior Malpighian tubules excluding stellate cells.
 - c) The reabsorptive lower tubule and ureter are also included in the GAL4 expression together with the main segment.
 - d) A view of the main segment of anterior Malpighian tubules.
 - e,f) GAL4 is expressed in the bar-shaped stellate cells of initial and transitional segment.
 - g) For comparison and confirmation of the expression pattern, the same expression detected with antibody against for β -gal was given.
 - h) The bar-shaped stellate cells at higher magnification.
-

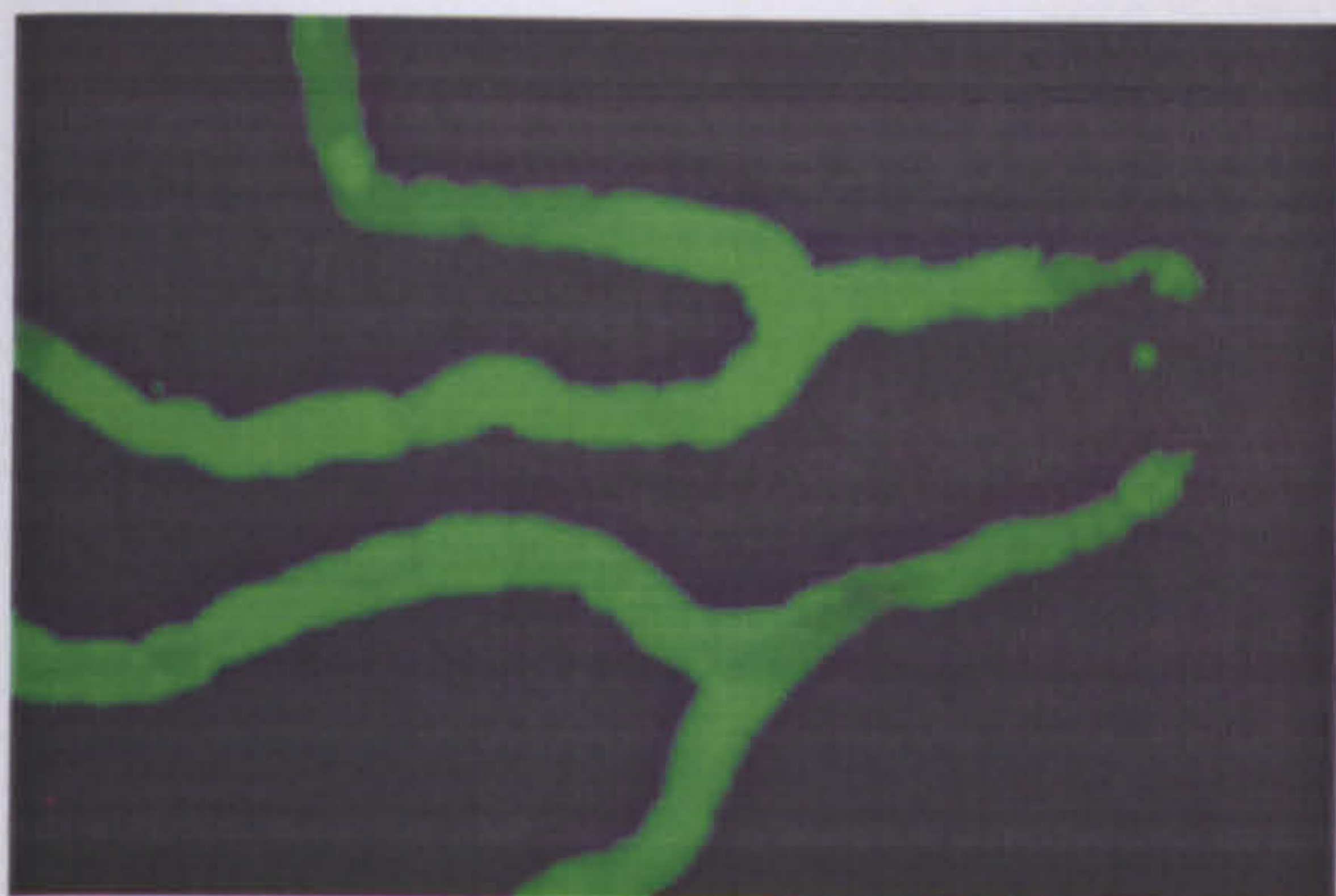
a)



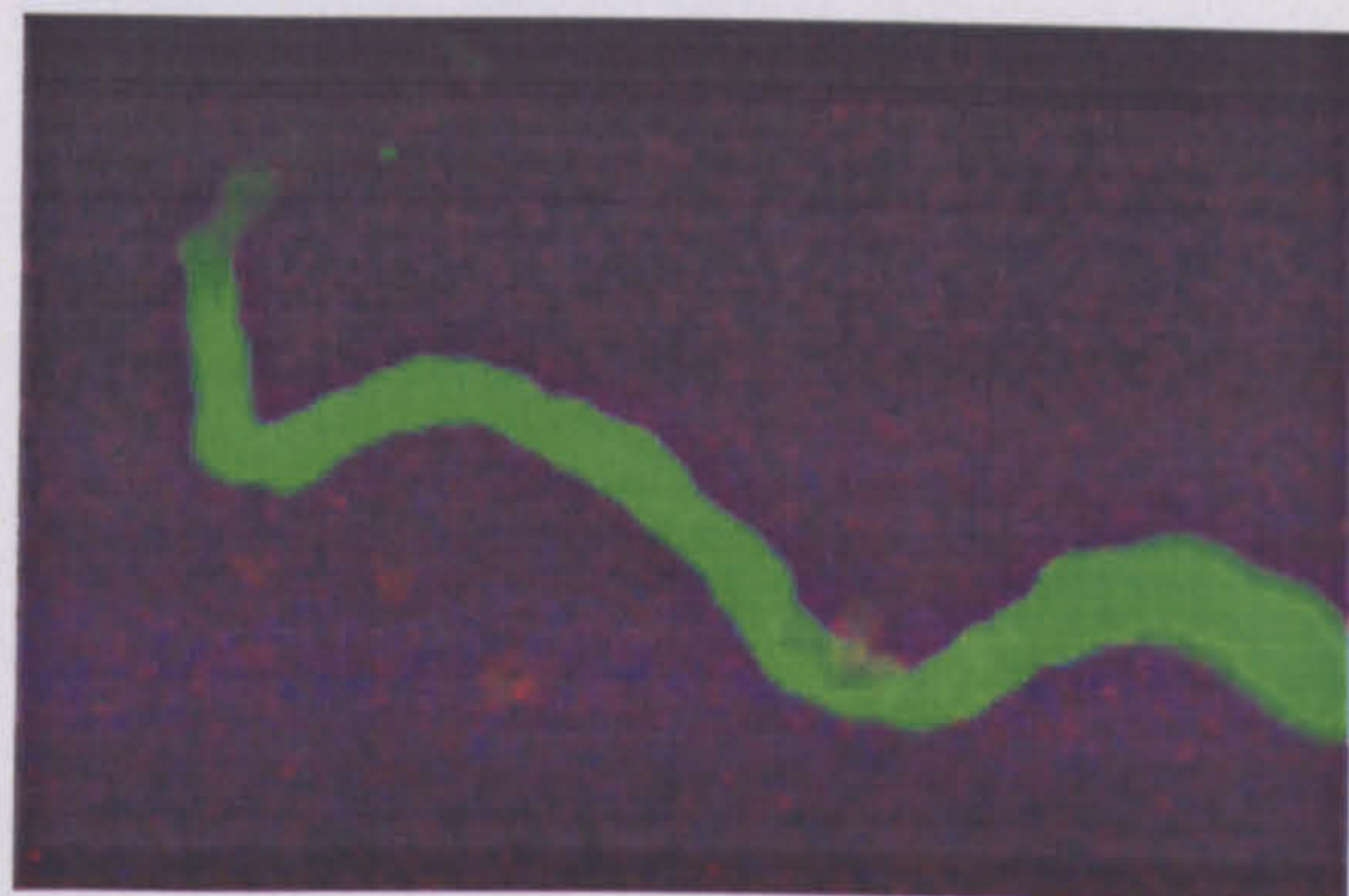
b)



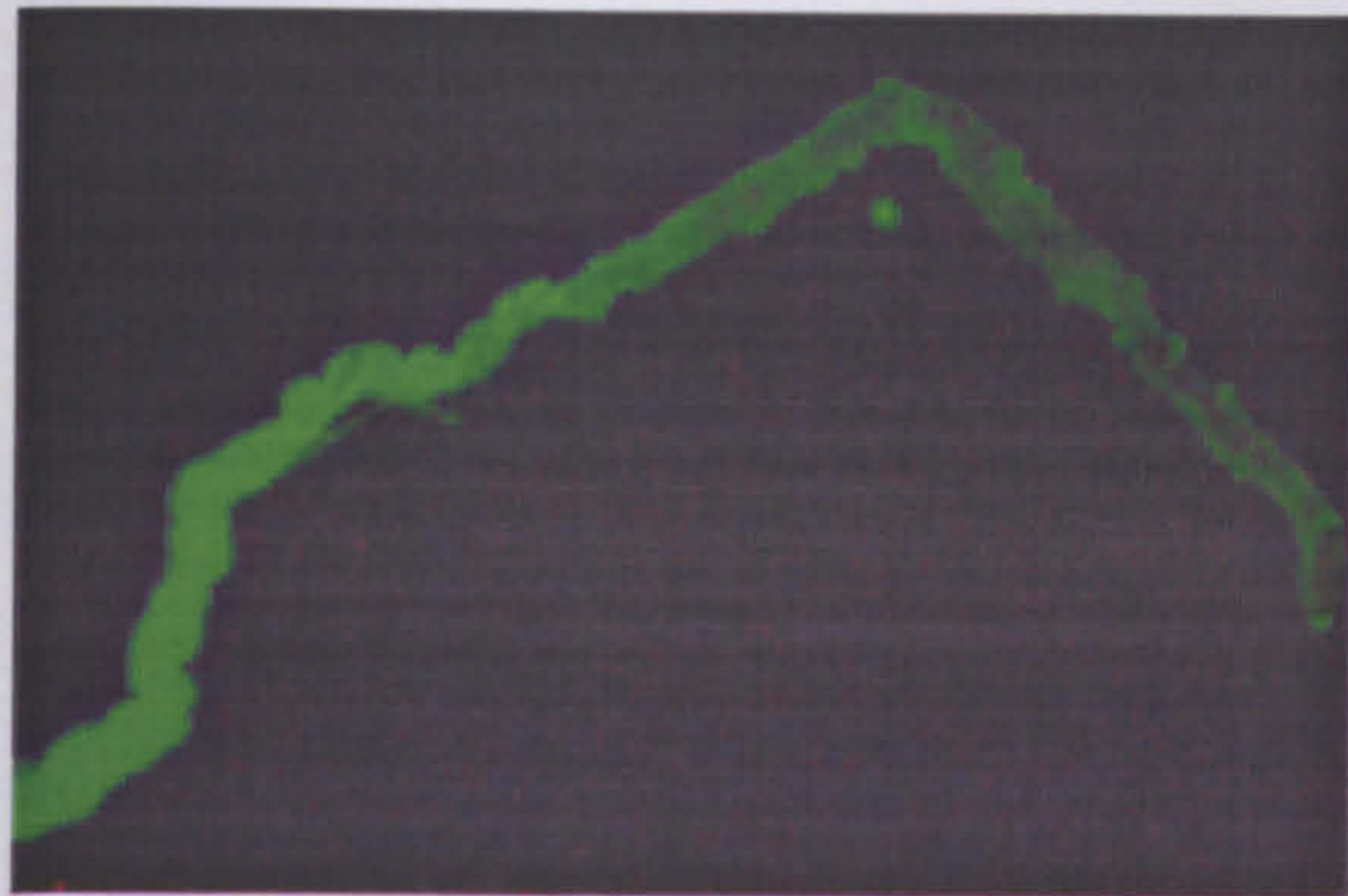
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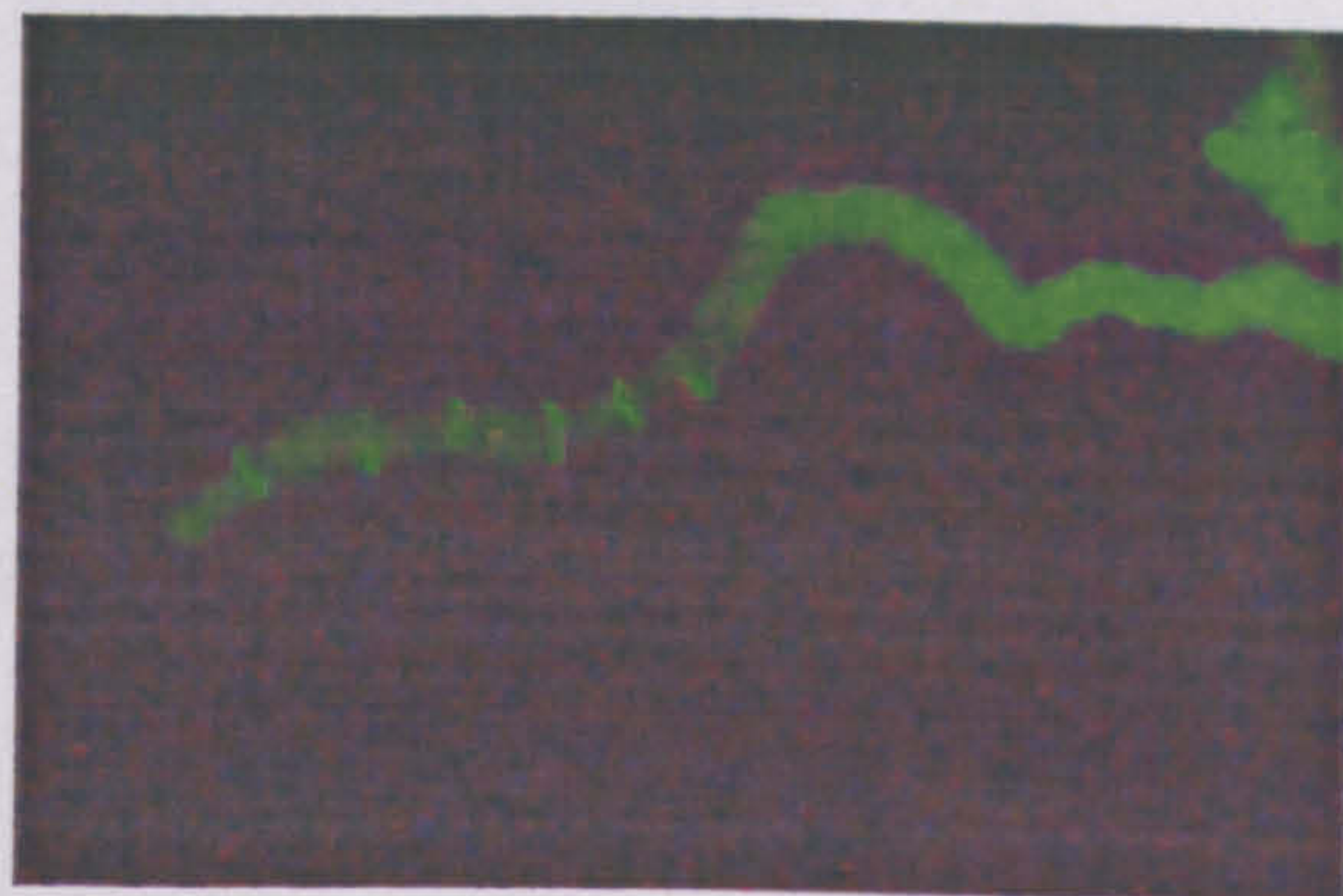
d)



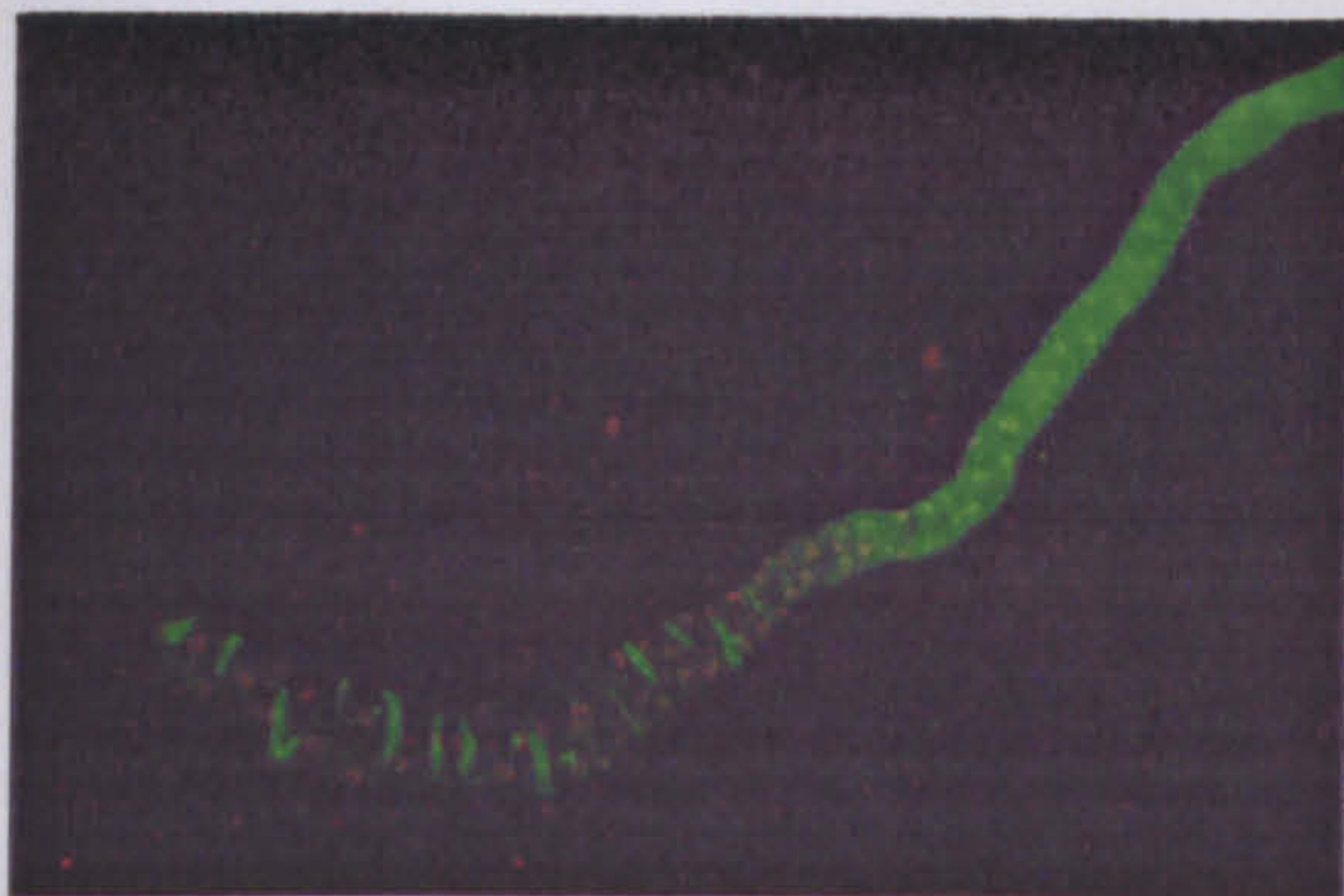
e)



f)



g)



h)

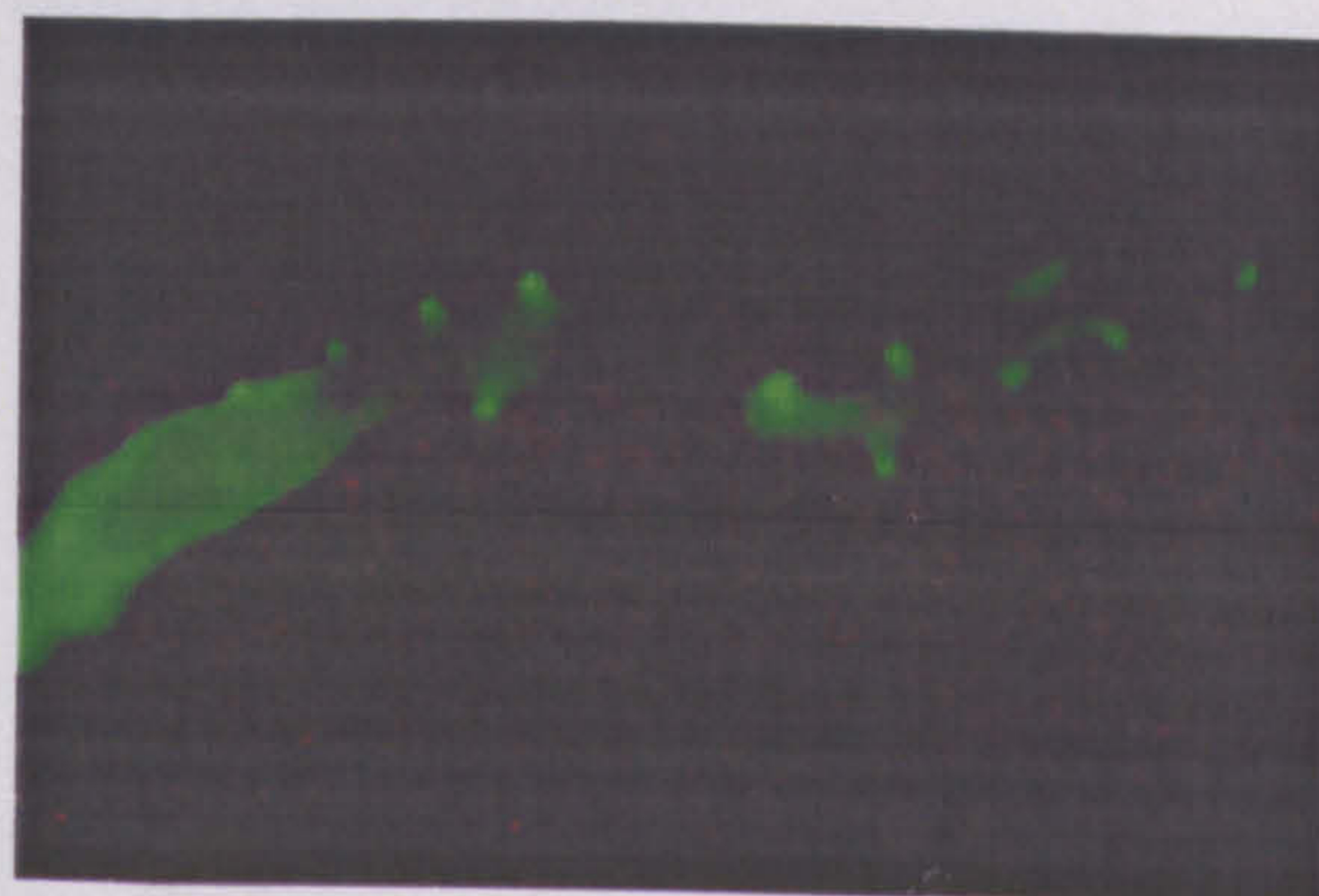


Figure 3.17. Line 709. GAL4-directed GFP expression in the adult Malpighian tubules by epifluorescence.

a,b) GAL4 expression in the anterior Malpighian tubules

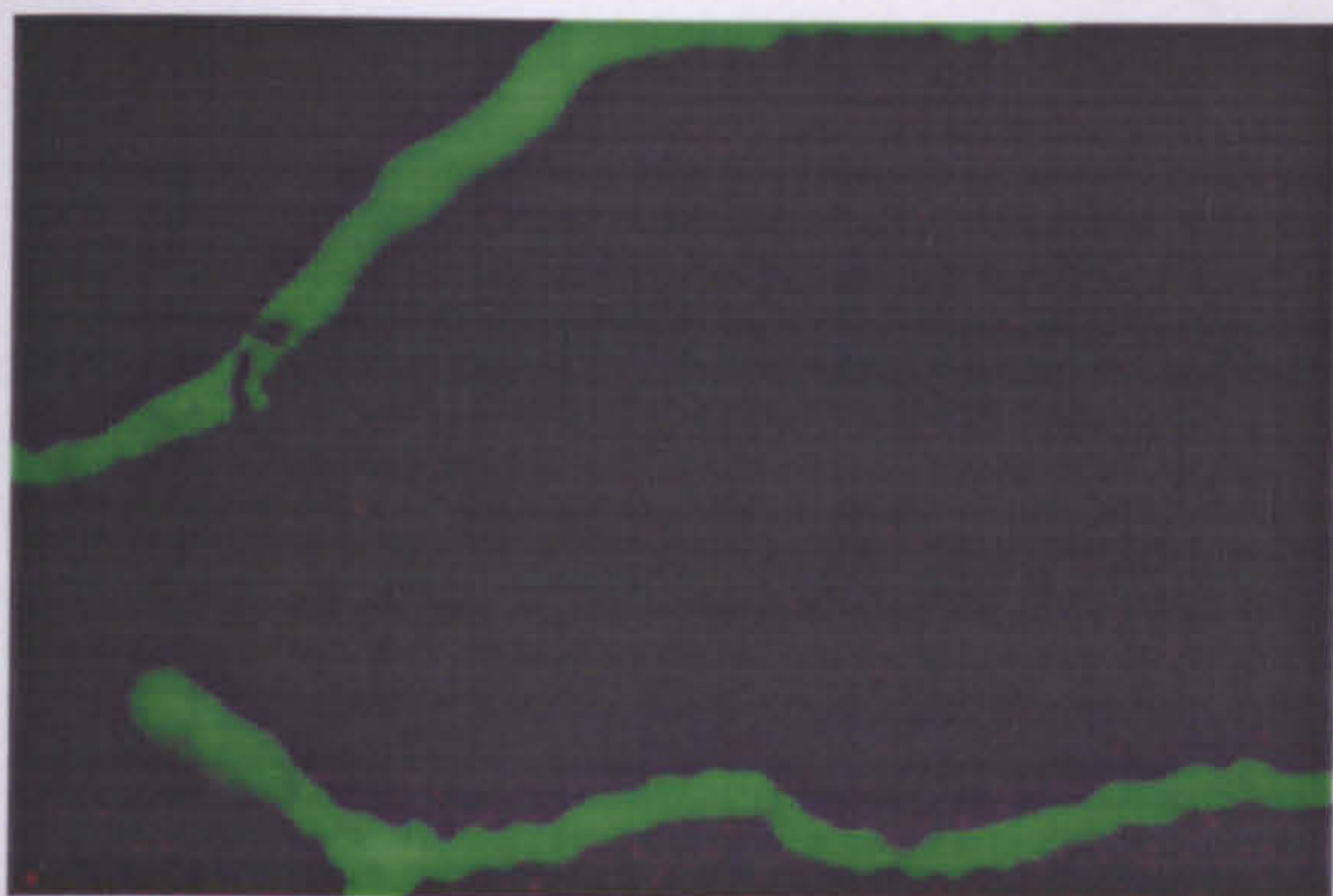
c,d) Expression is confined to the principal cells, excluding the stellate cells.

e,f) The non-staining stellate cells of main segment in the posterior and anterior Malpighian tubules respectively, at higher magnification.

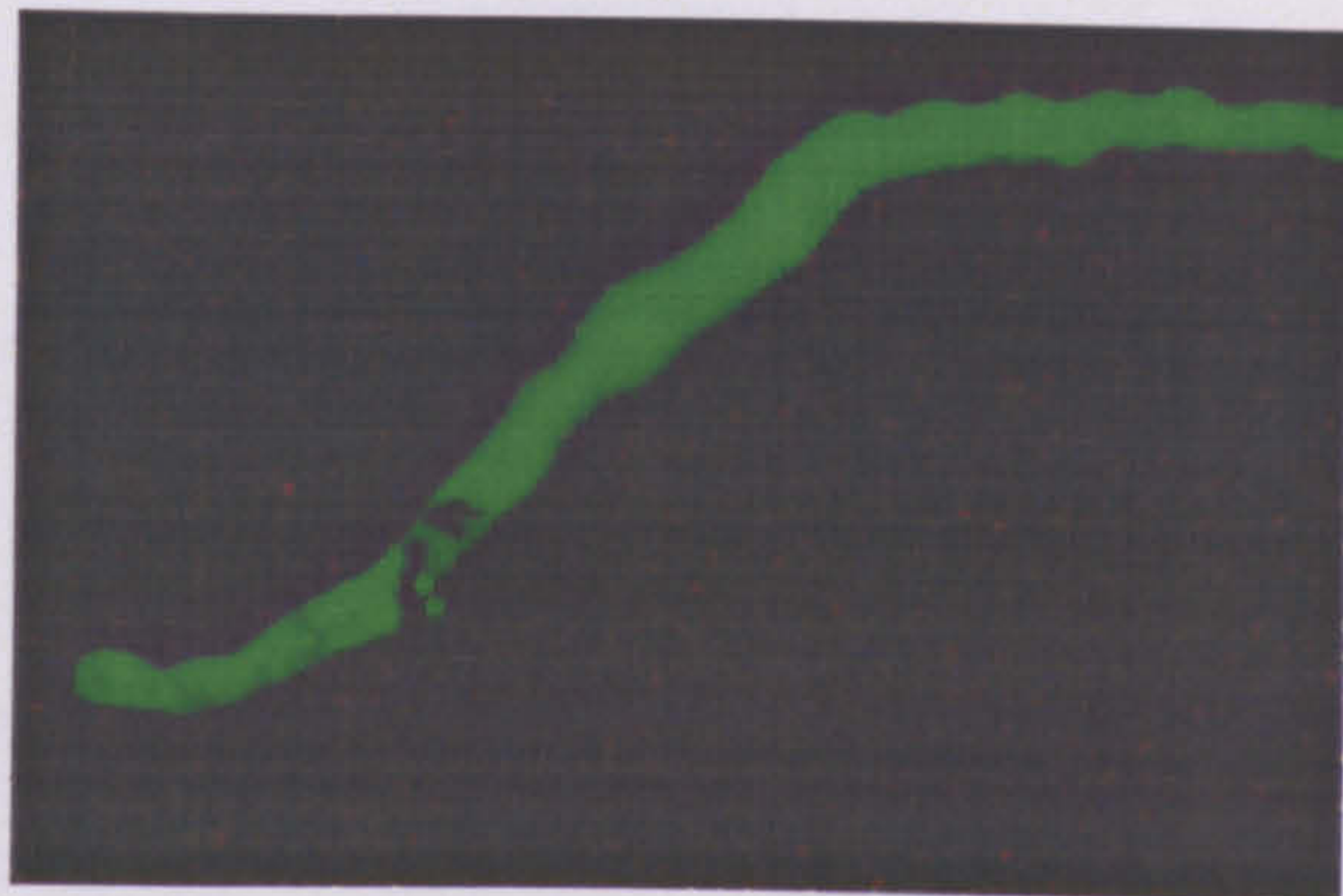
g) Contrary to the previous line (c42), in this line bar-shaped stellate cells do not have any detectable expression.

h) The equivalent of the non-transporting region of the distal part in the posterior Malpighian tubules showing no detectable expression.

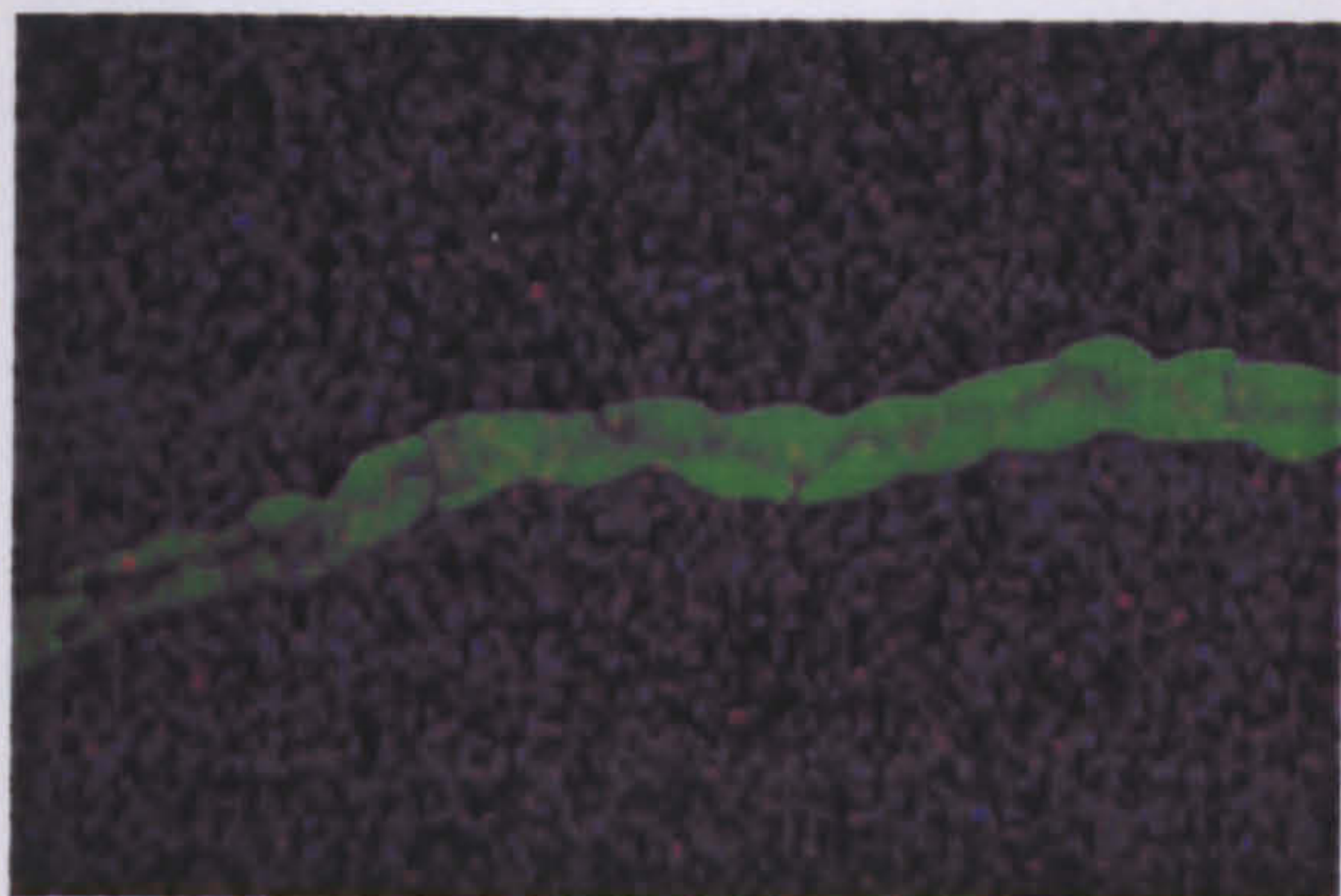
a)



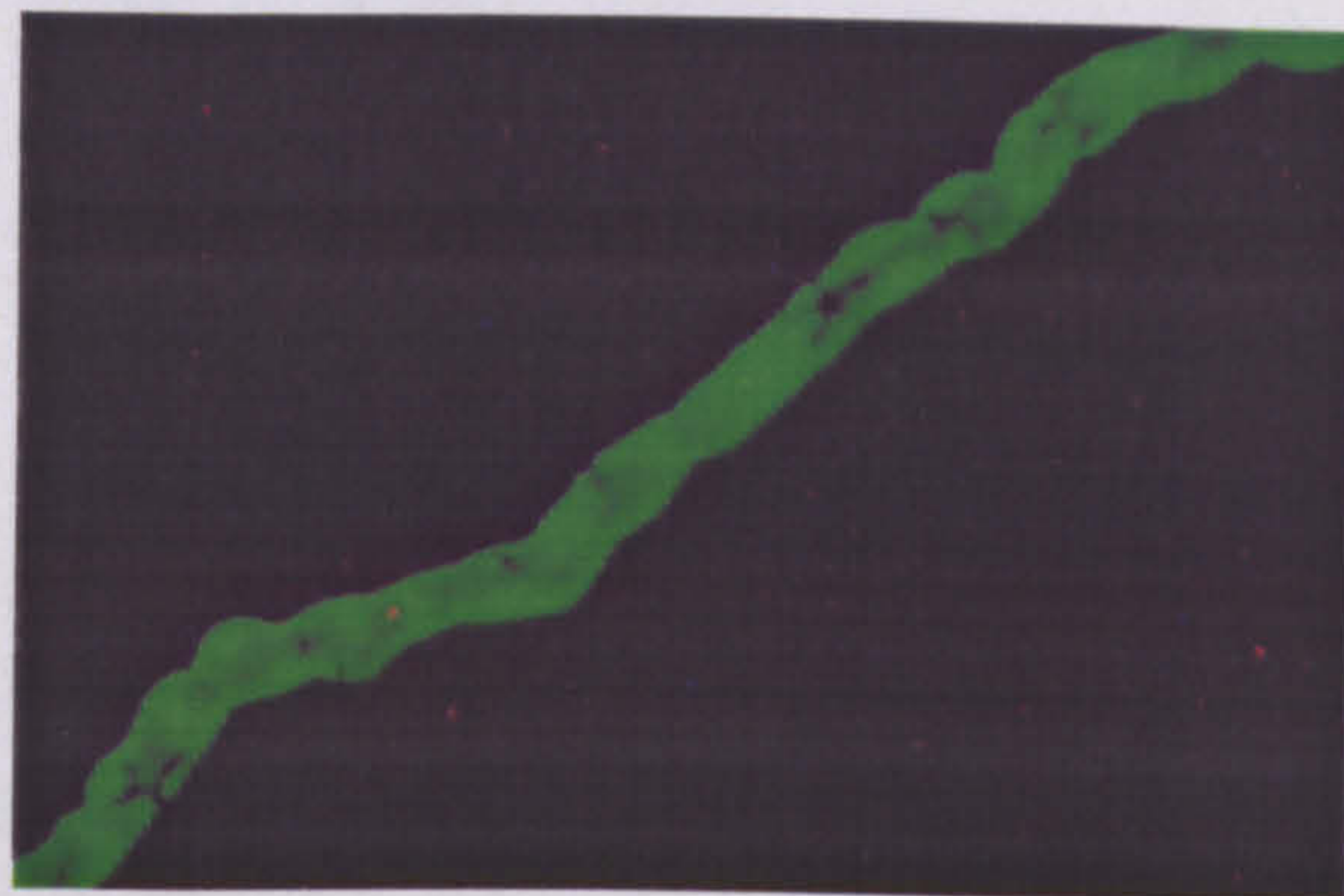
b)



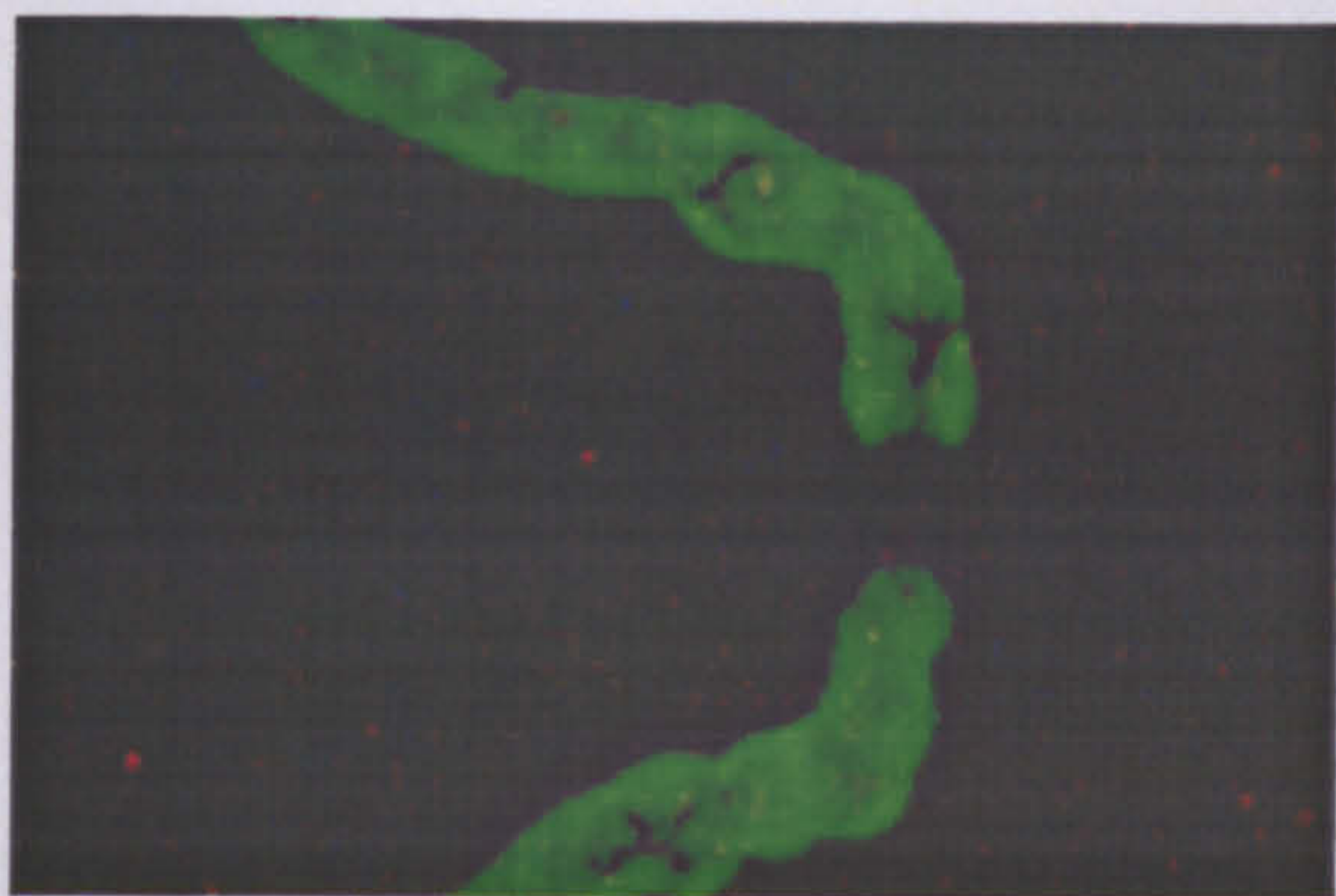
c)



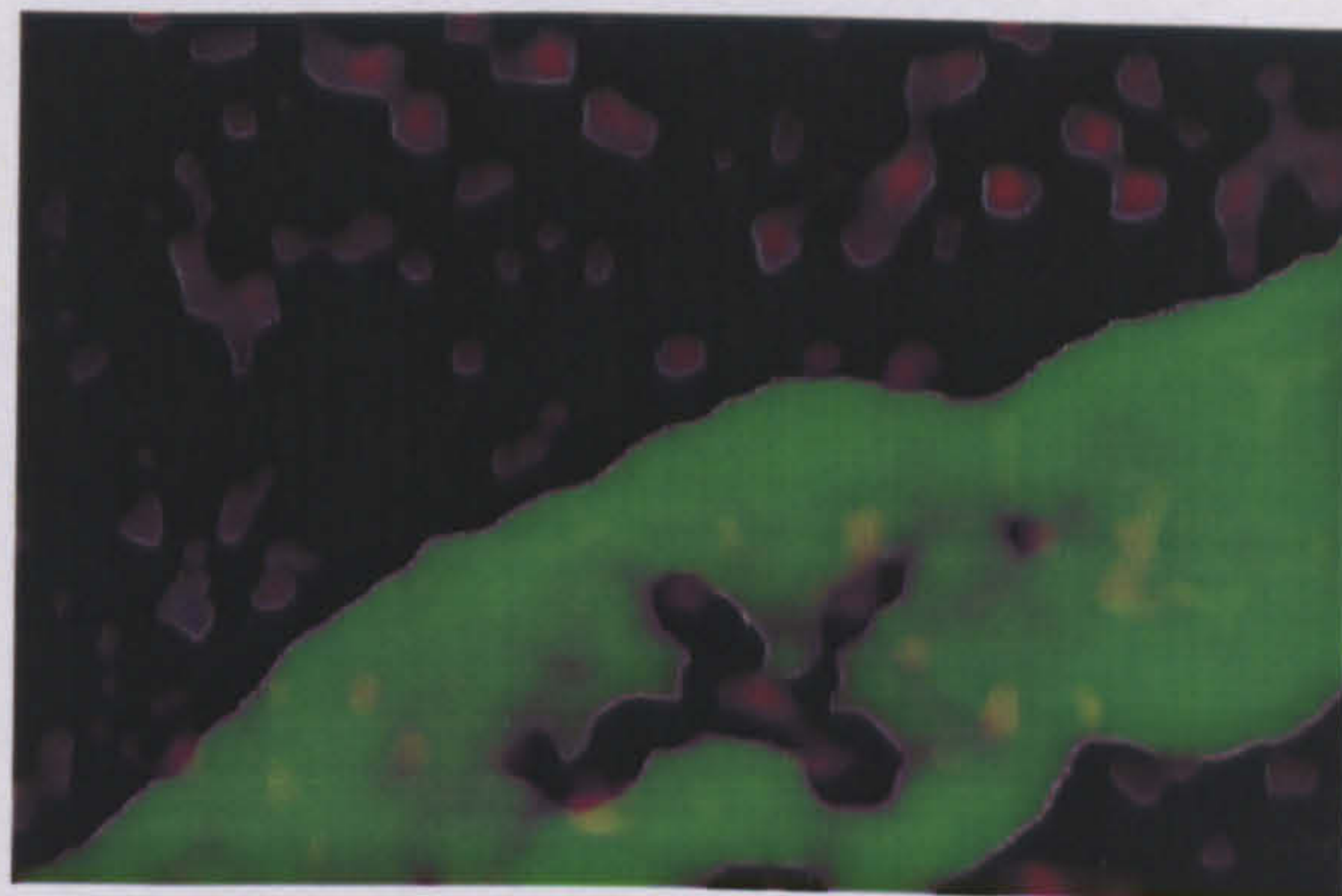
d)



e)



f)



g)



h)

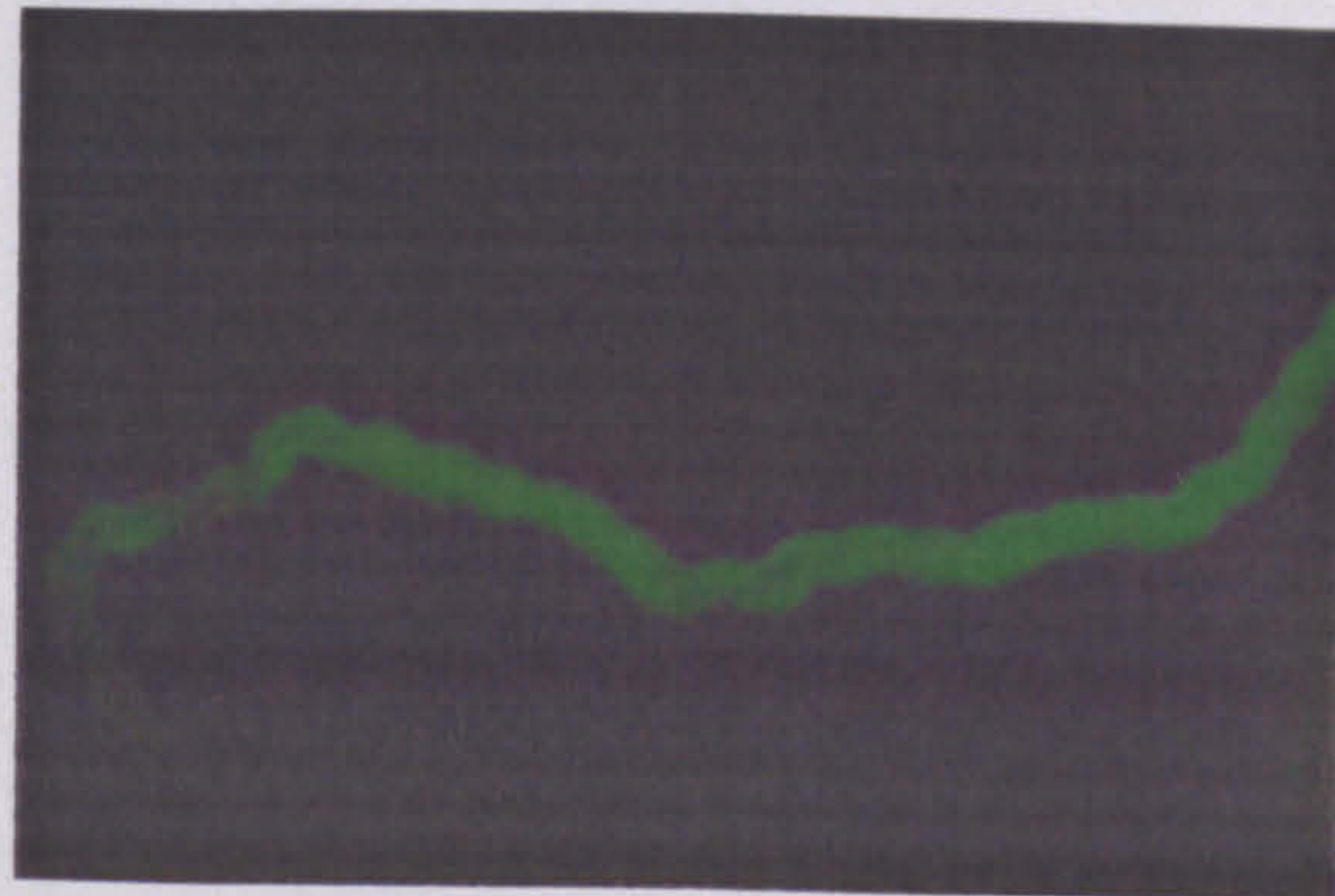


Figure 3.18. Line c724. Larval (third instar) GAL4-directed GFP expression patterns of Malpighian tubules by epifluorescence.

- a) GAL4 is expressed in all stellate cells of anterior Malpighian tubules as well as posterior ones.
 - b) Three stellate cells equivalent to the bar-shaped ones in the distal part (initial and transitional segments) of adult anterior Malpighian tubules in higher resolution.
 - c) A single bar-shaped cell at higher magnification.
 - d) Several stellate cells equivalent to the stellate-shaped ones in the main segment of anterior Malpighian tubules at higher magnification.
 - e) A single stellate cell from main segment at higher magnification.
 - f) The overall view of stellate cells of posterior Malpighian tubules.
 - g) Some stellate cells from posterior Malpighian tubules at higher magnification.
 - h) A stellate cell from main segment of posterior Malpighian tubules at higher magnification.
-

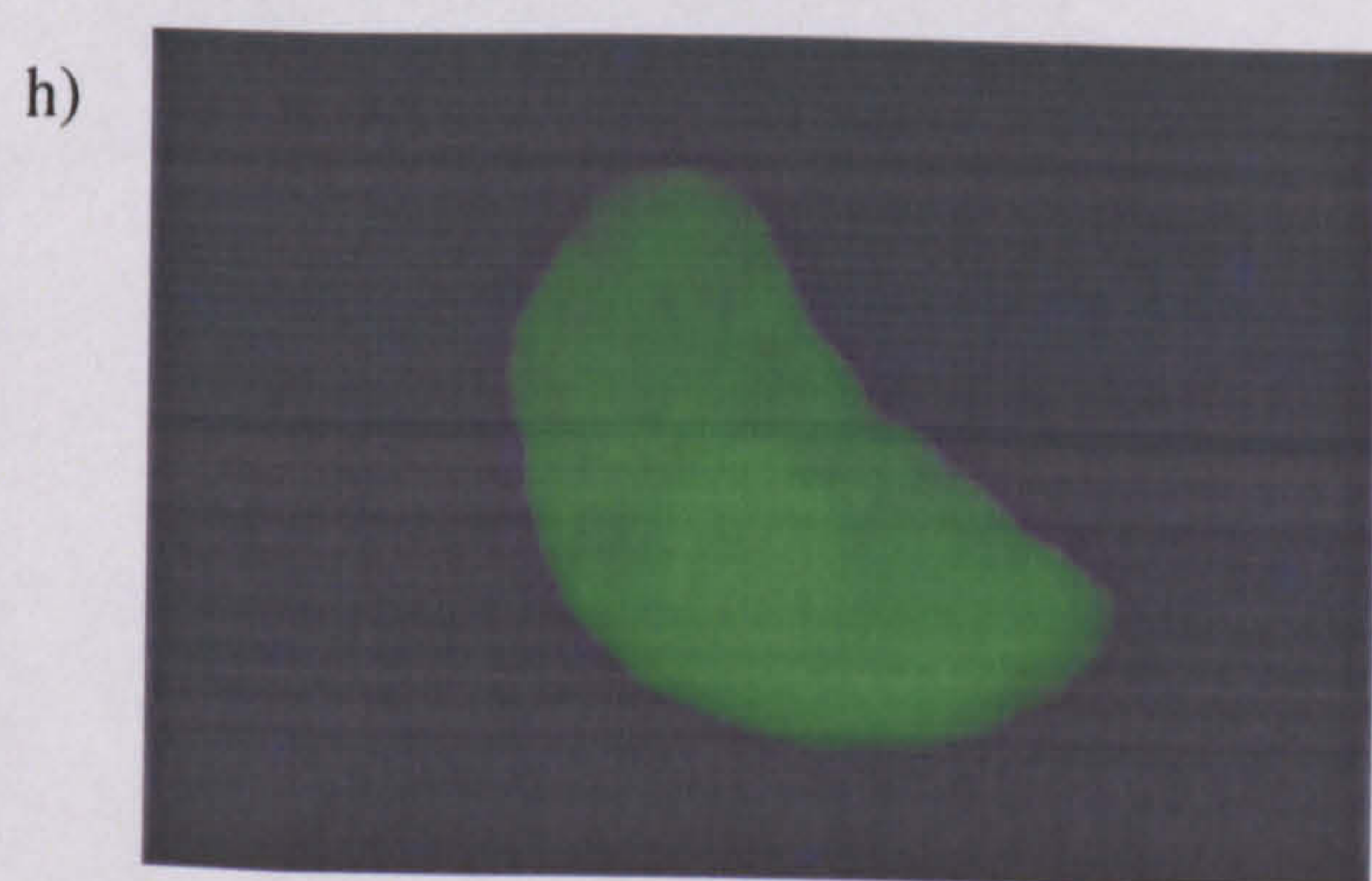
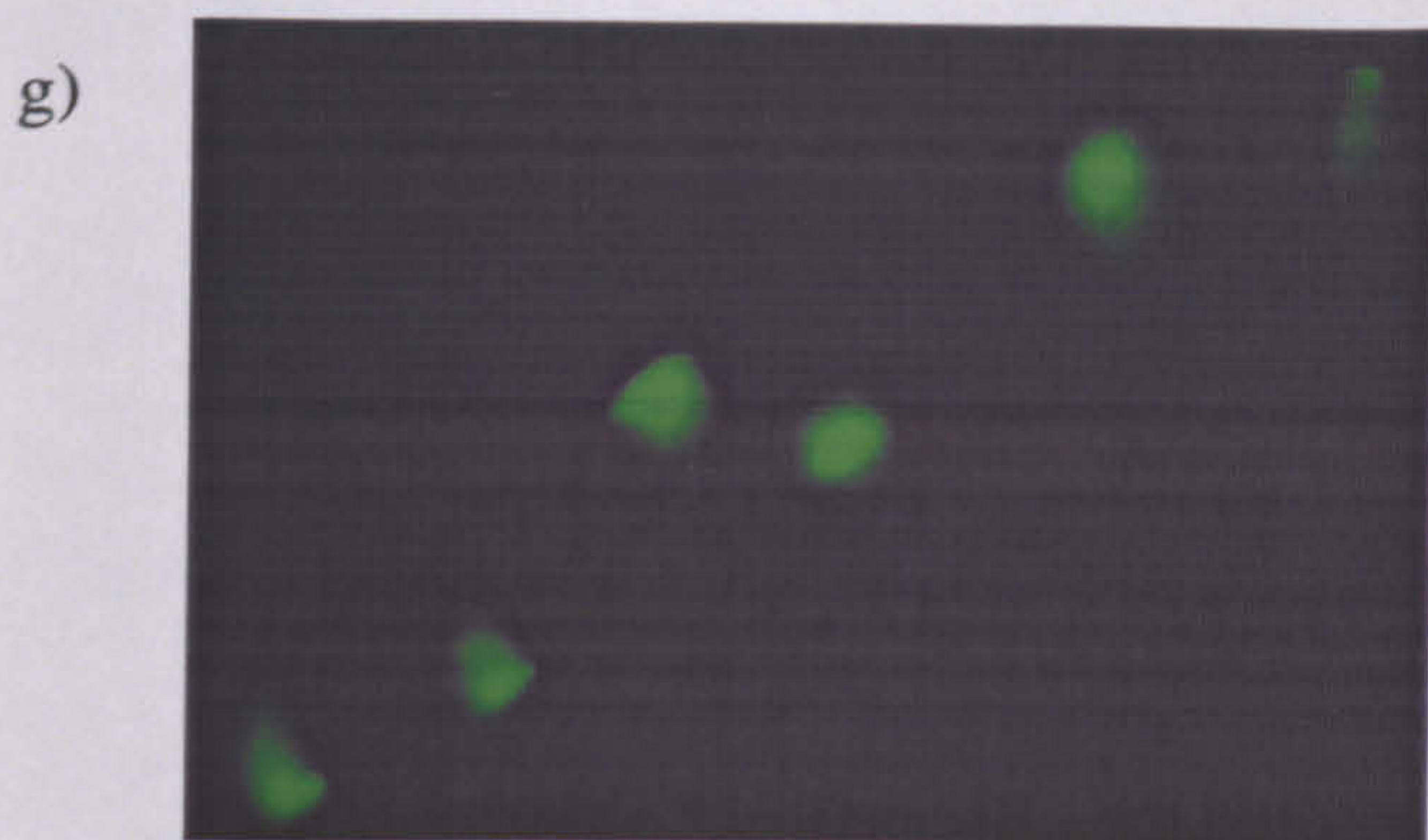
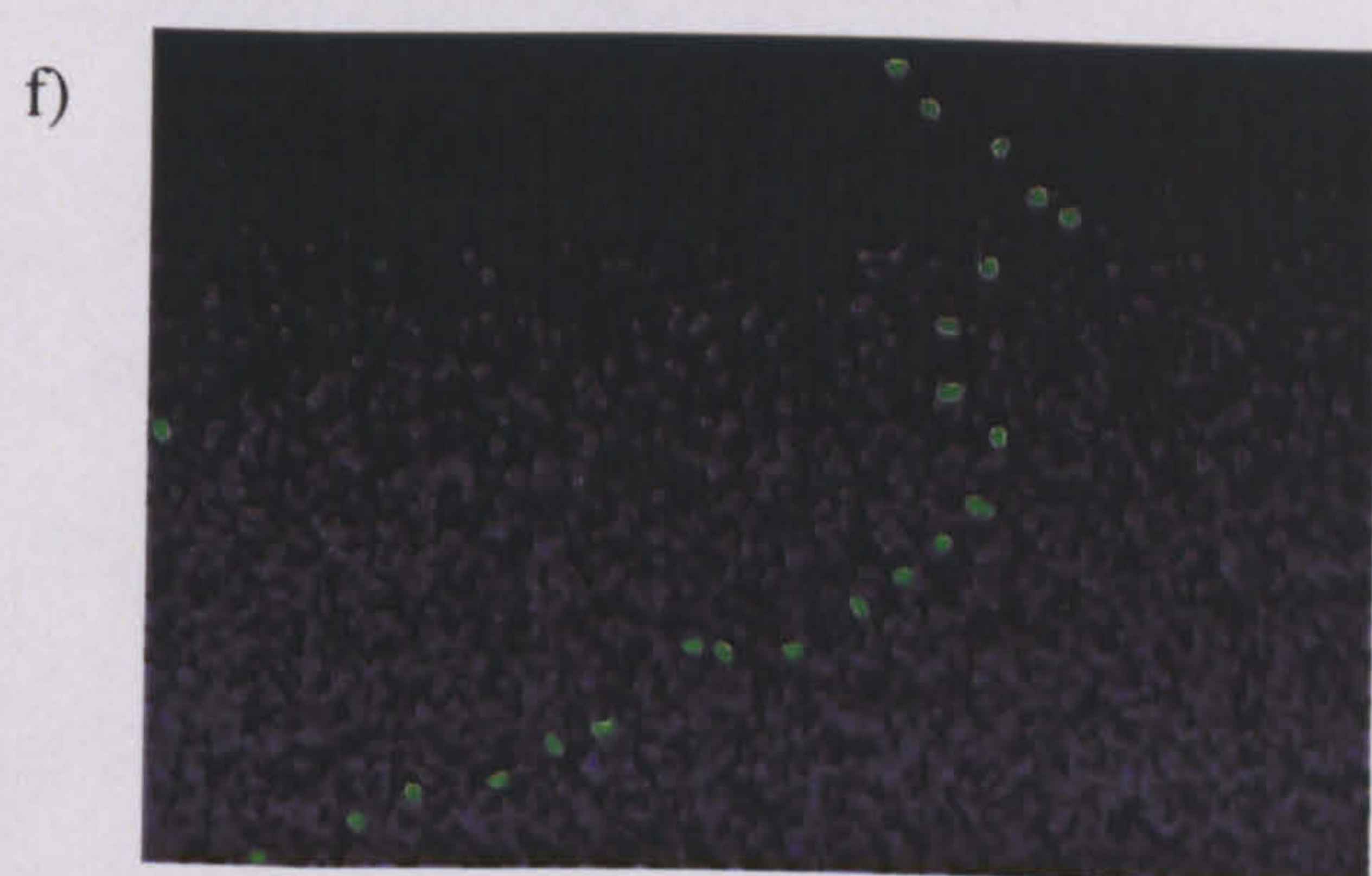
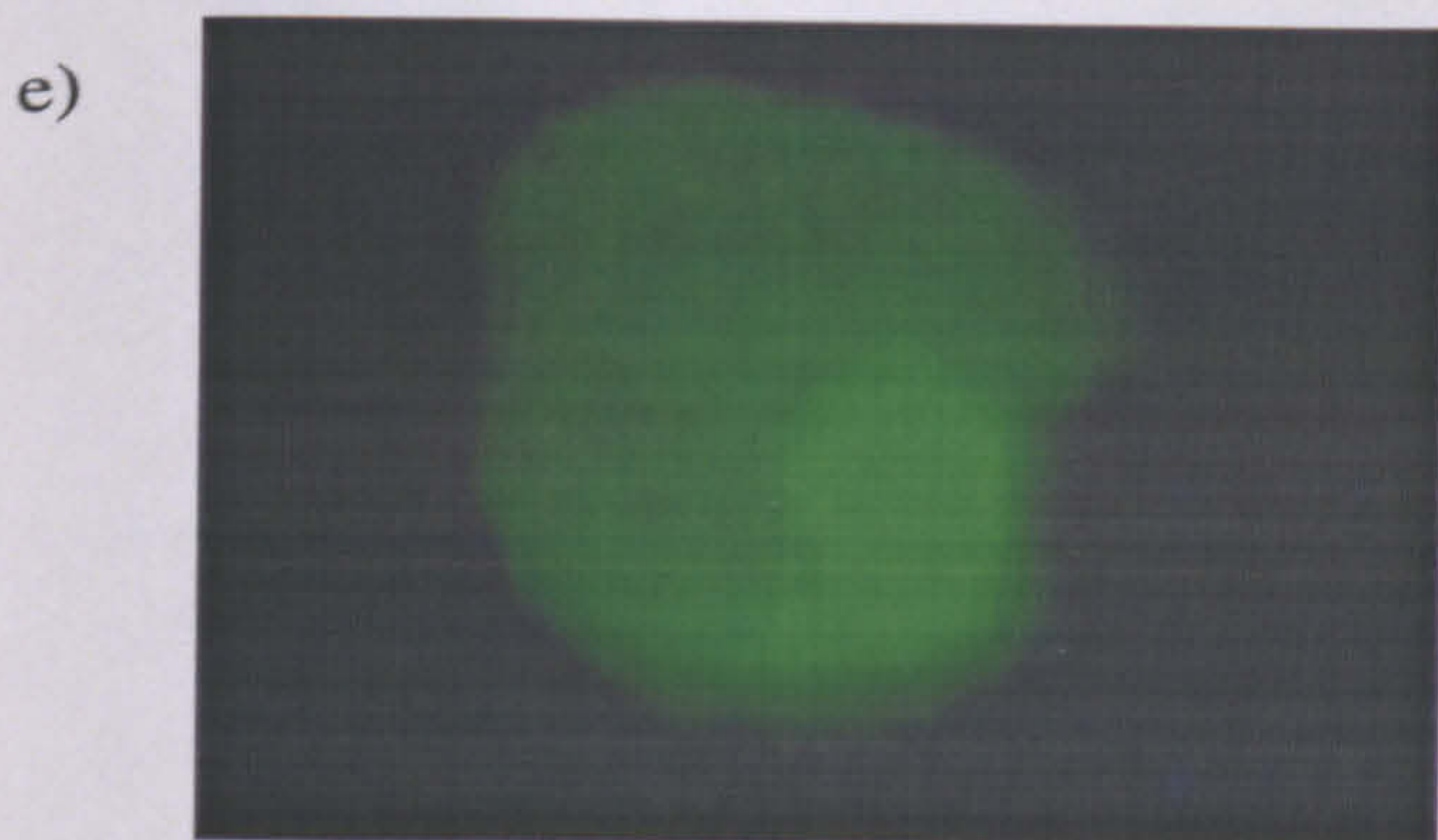
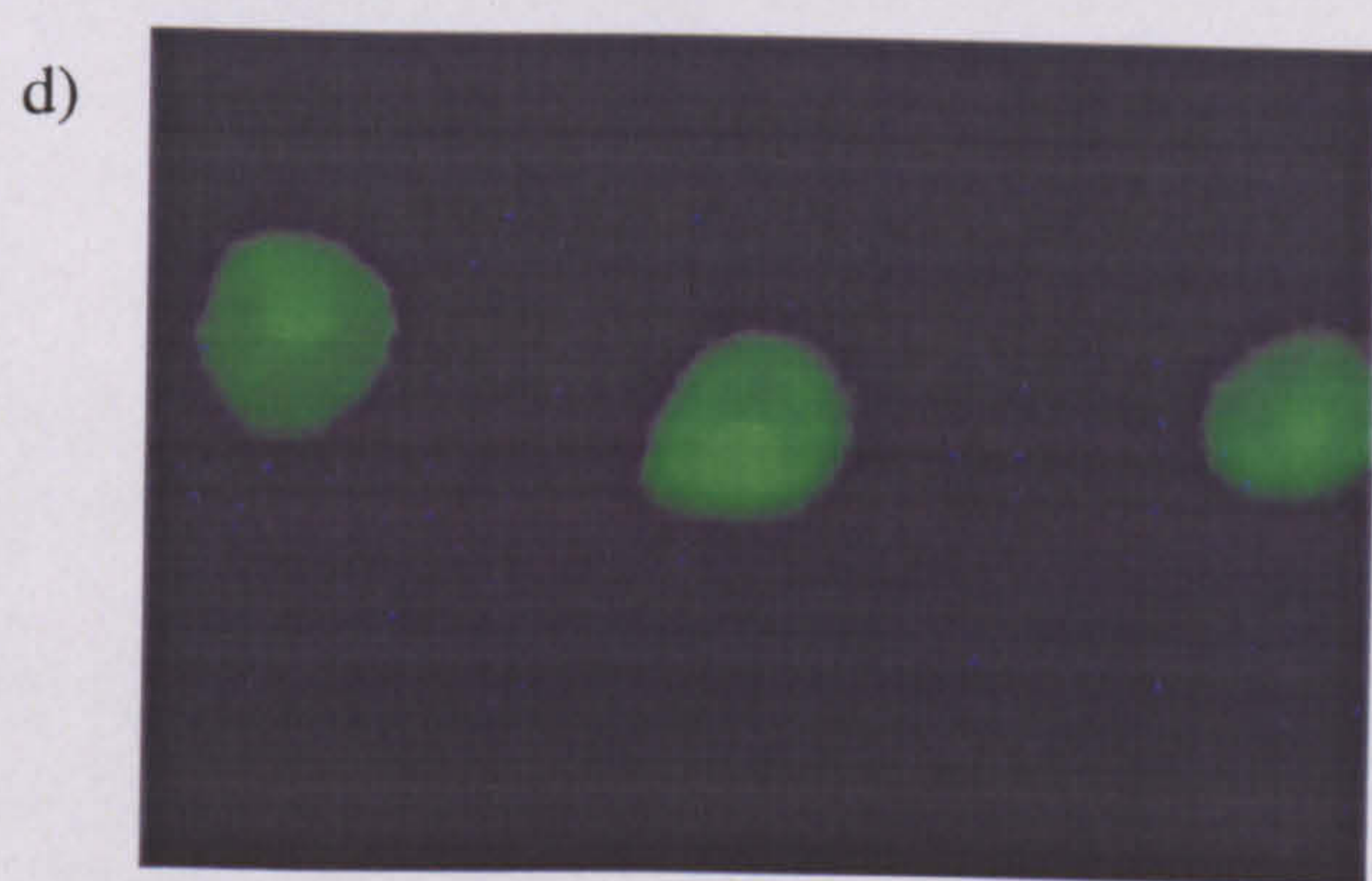
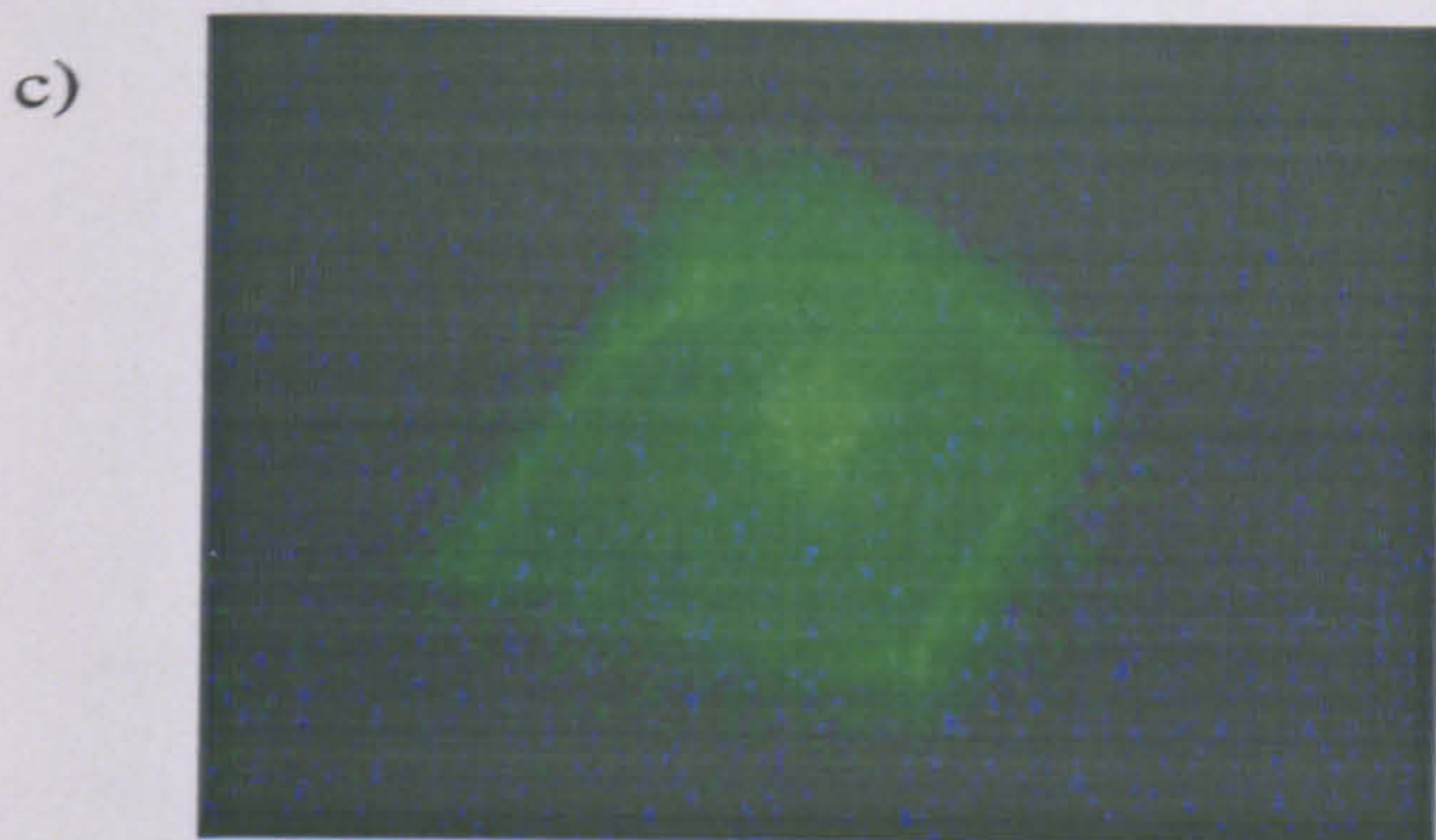
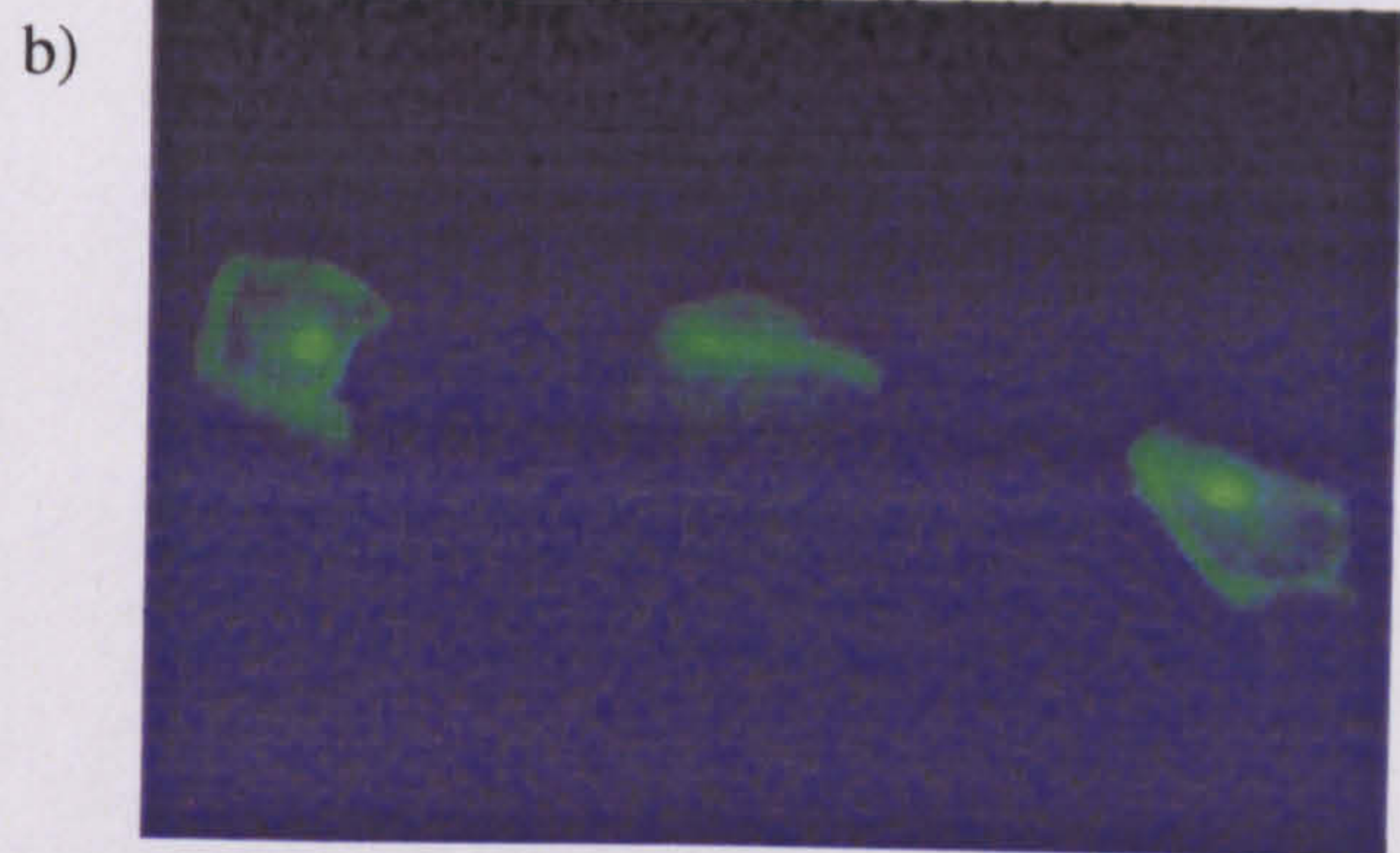


Figure 3.19. Line c710. Larval GAL4-directed GFP expression patterns of Malpighian tubules by epifluorescence.

- a) GAL4 expression is in all stellate cells of main segment in the anterior Malpighian tubules as well as posterior ones, but is not detectable in those of the distal part of anterior Malpighian tubules.
 - b) GAL4 expression boundary in the anterior Malpighian tubules. Though expression can be seen in the main segment stellate cells, it can not be detected in type II cells in the distal part of the border.
 - c) Non-staining cells, which are probably equivalent to bar-shaped stellate cells of the initial and transitional segments, in larval anterior Malpighian tubules at higher magnification.
 - d) A single stellate cell from the main segment of anterior Malpighian tubules at higher magnification.
 - e) The overall view of GAL4 expression pattern in larval stellate cells of posterior Malpighian tubules.
 - f,g) Several stellate cells equivalent to the stellate-shaped ones in the main segment of anterior Malpighian tubules at higher magnification.
 - h) A single stellate cell from the posterior Malpighian tubules at higher magnification.
-

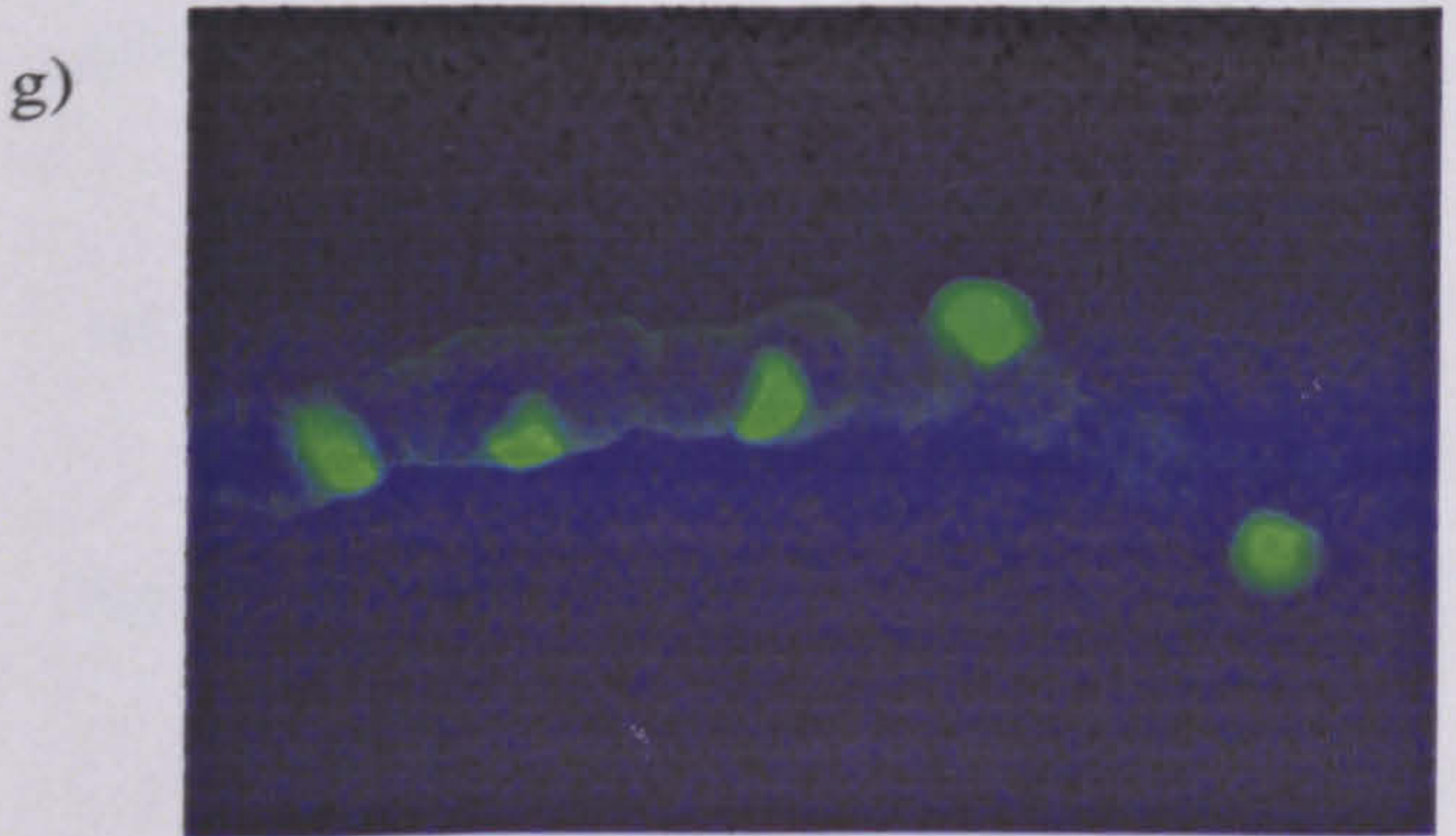
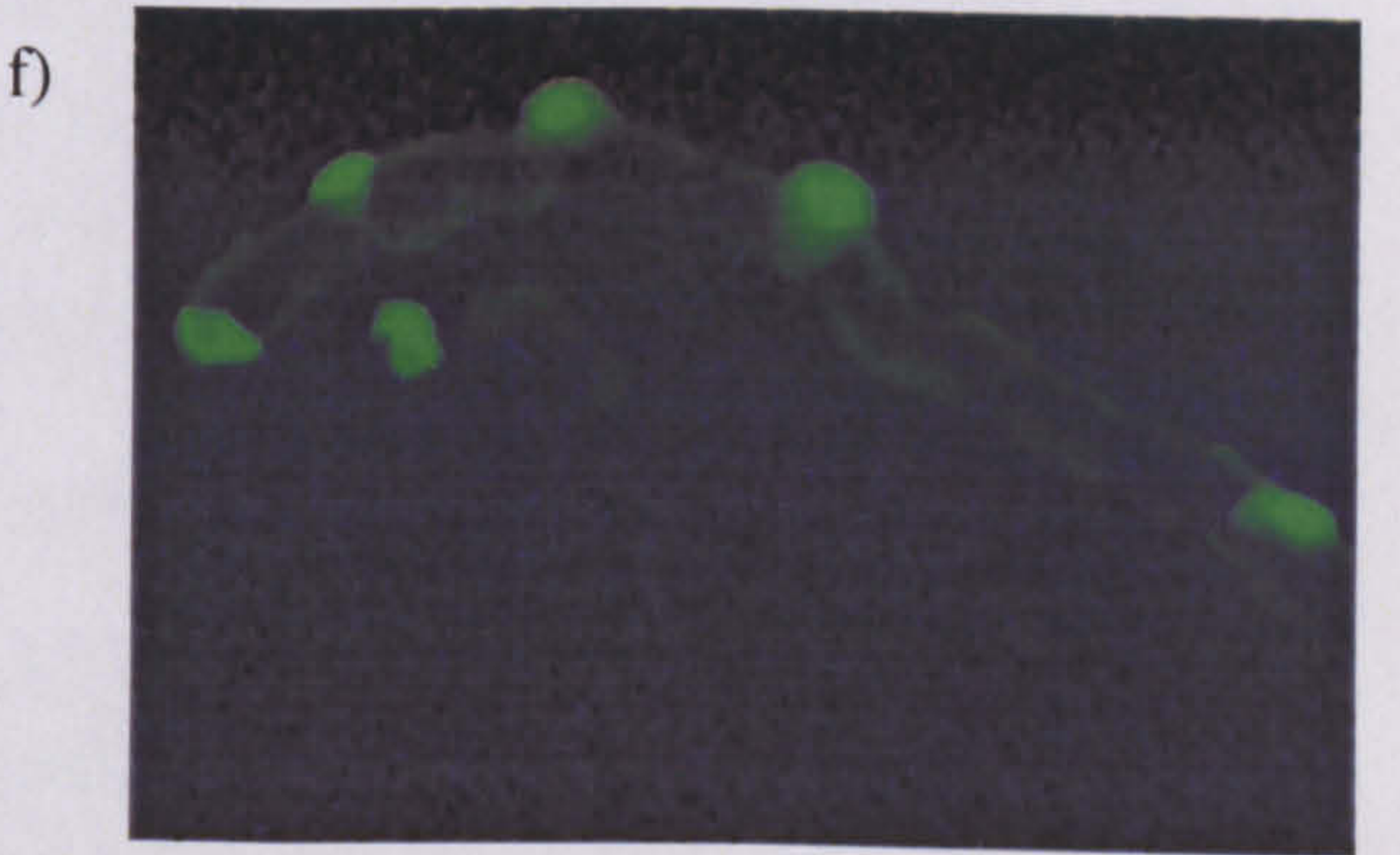
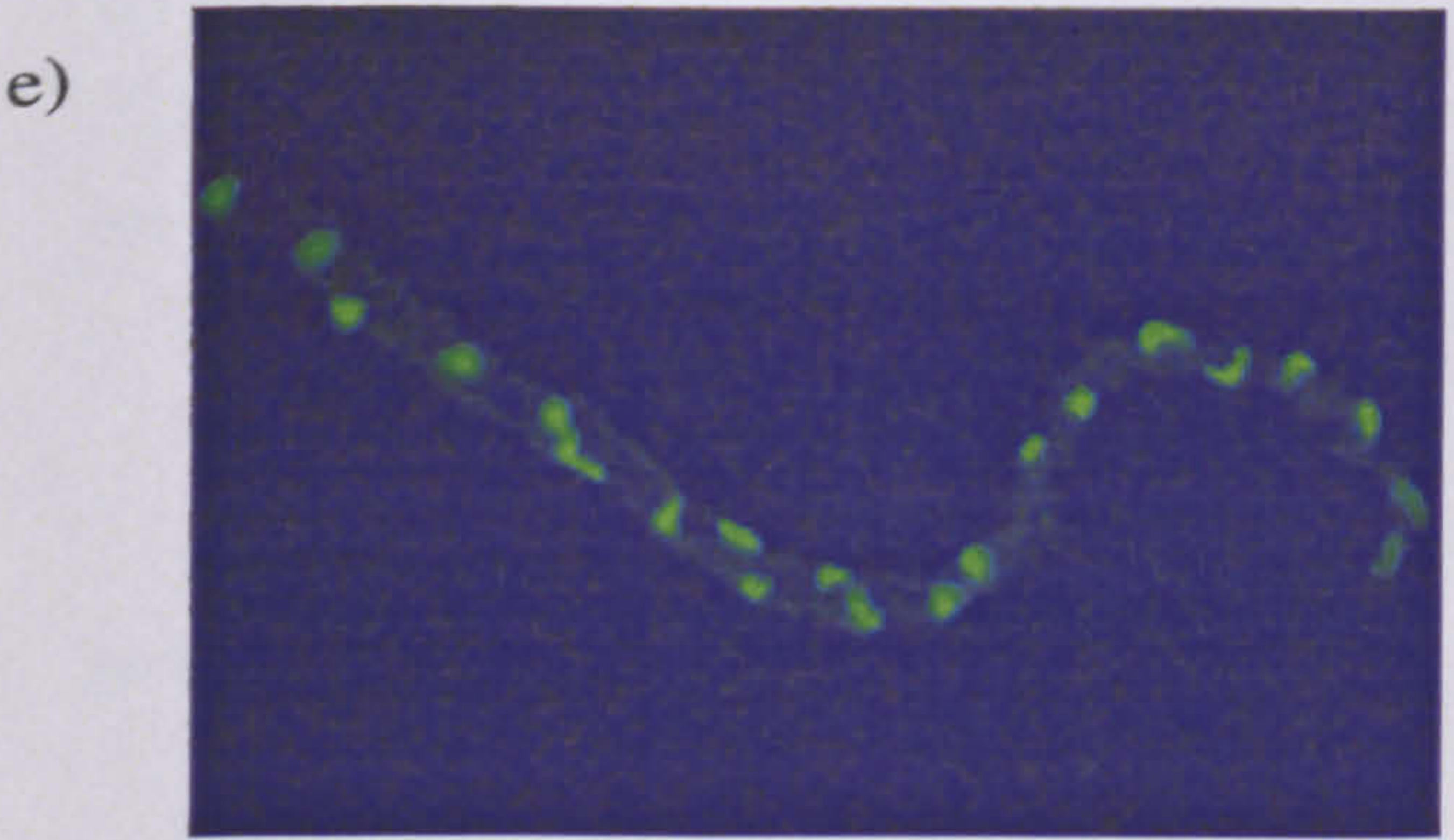
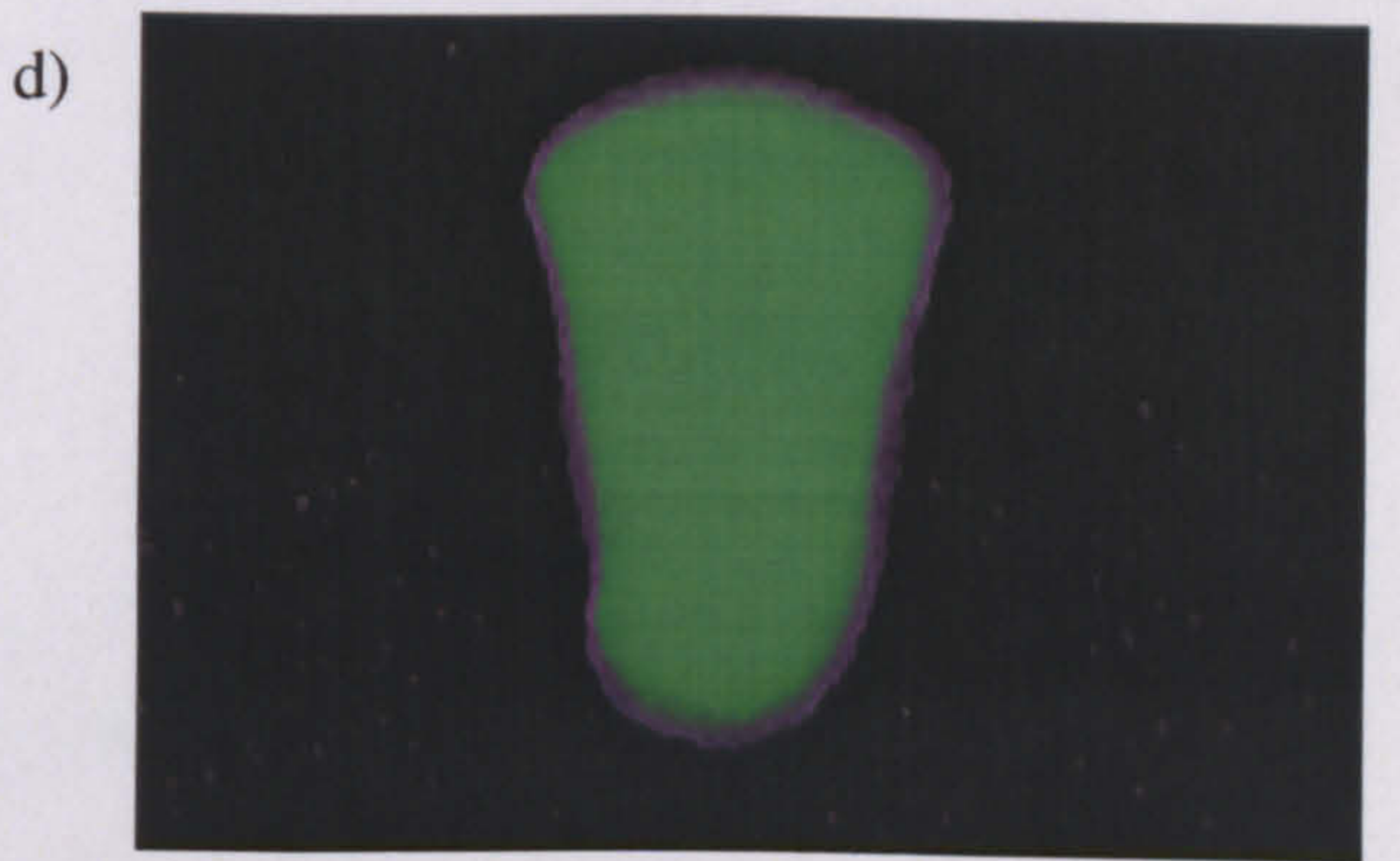
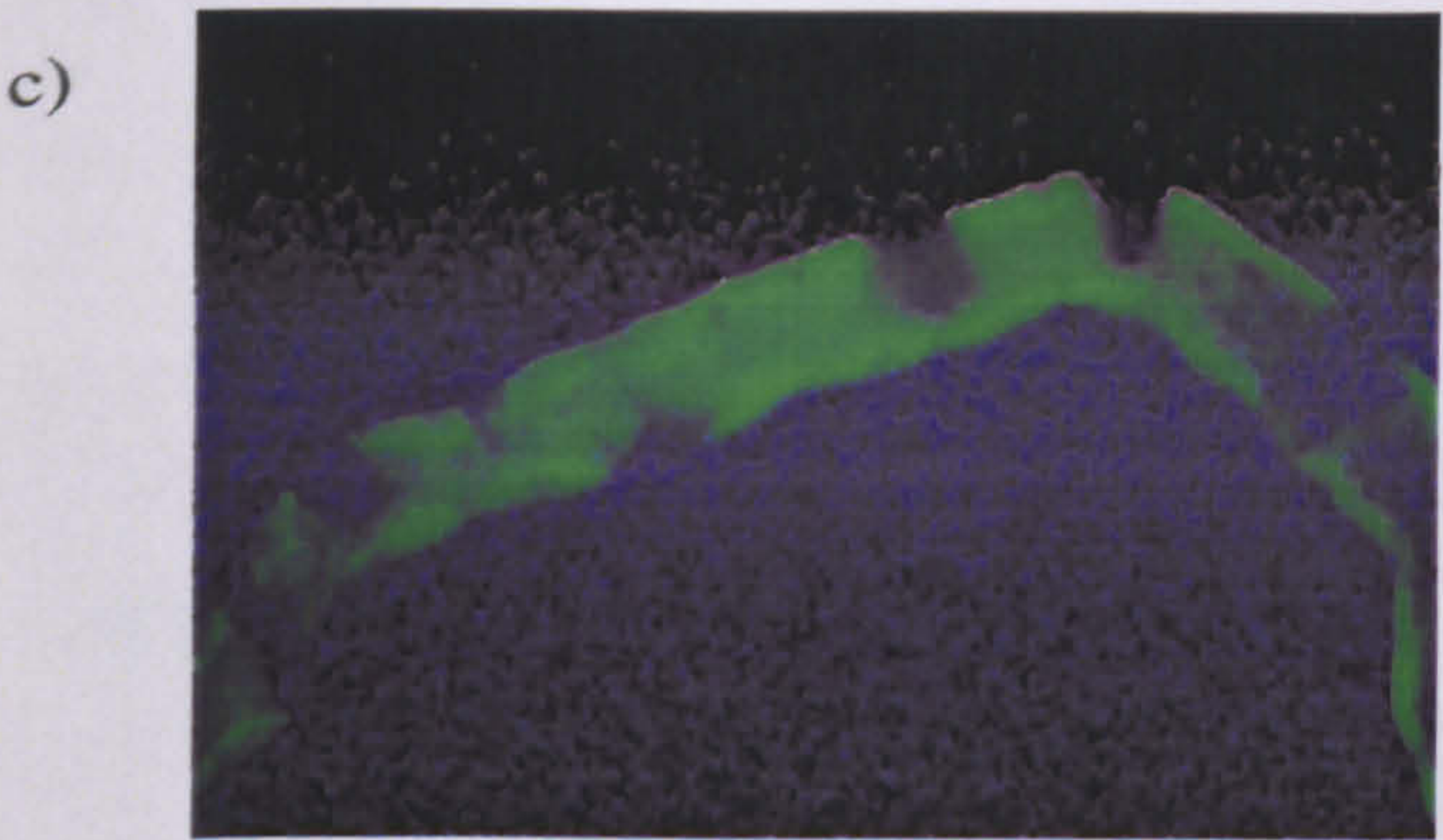
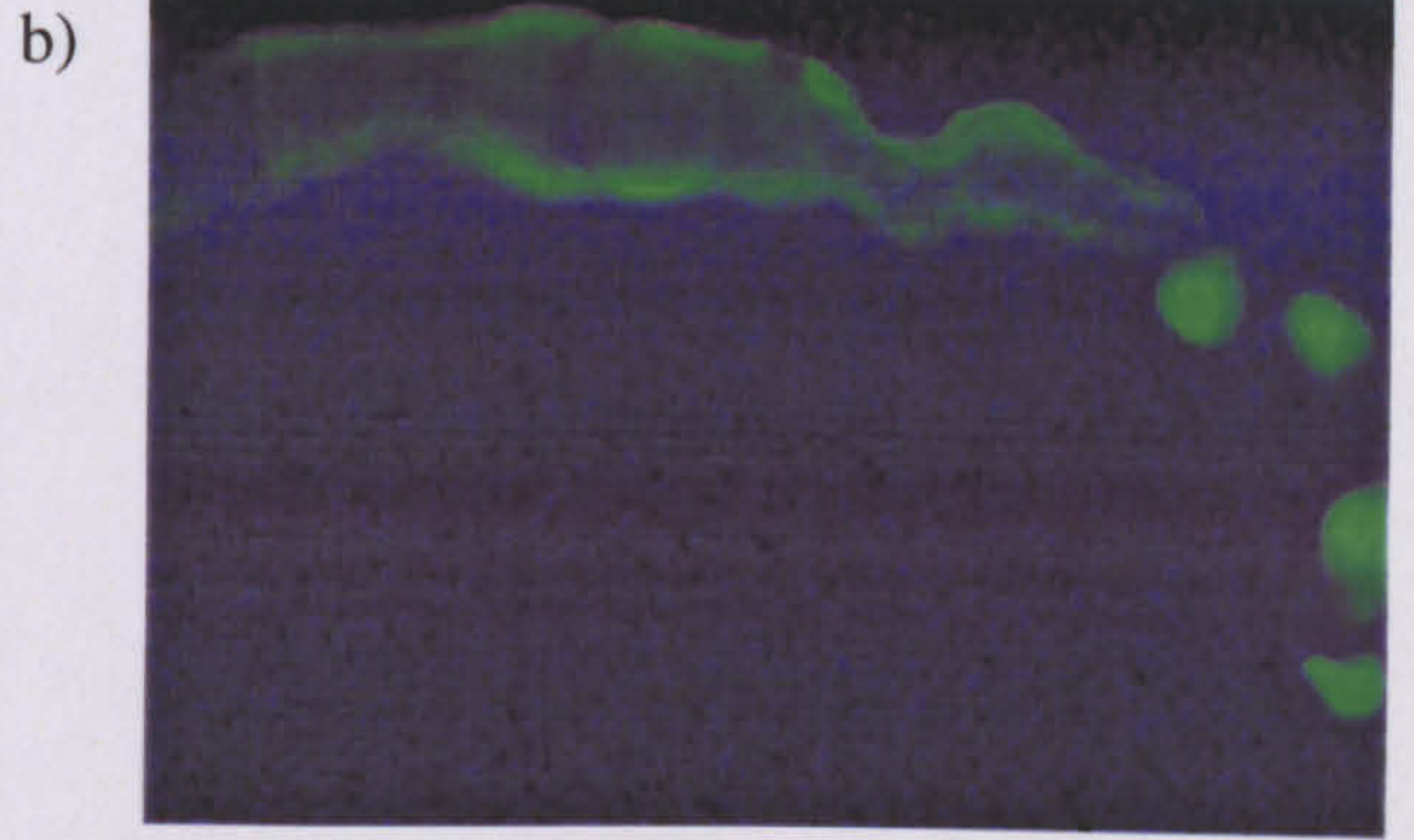
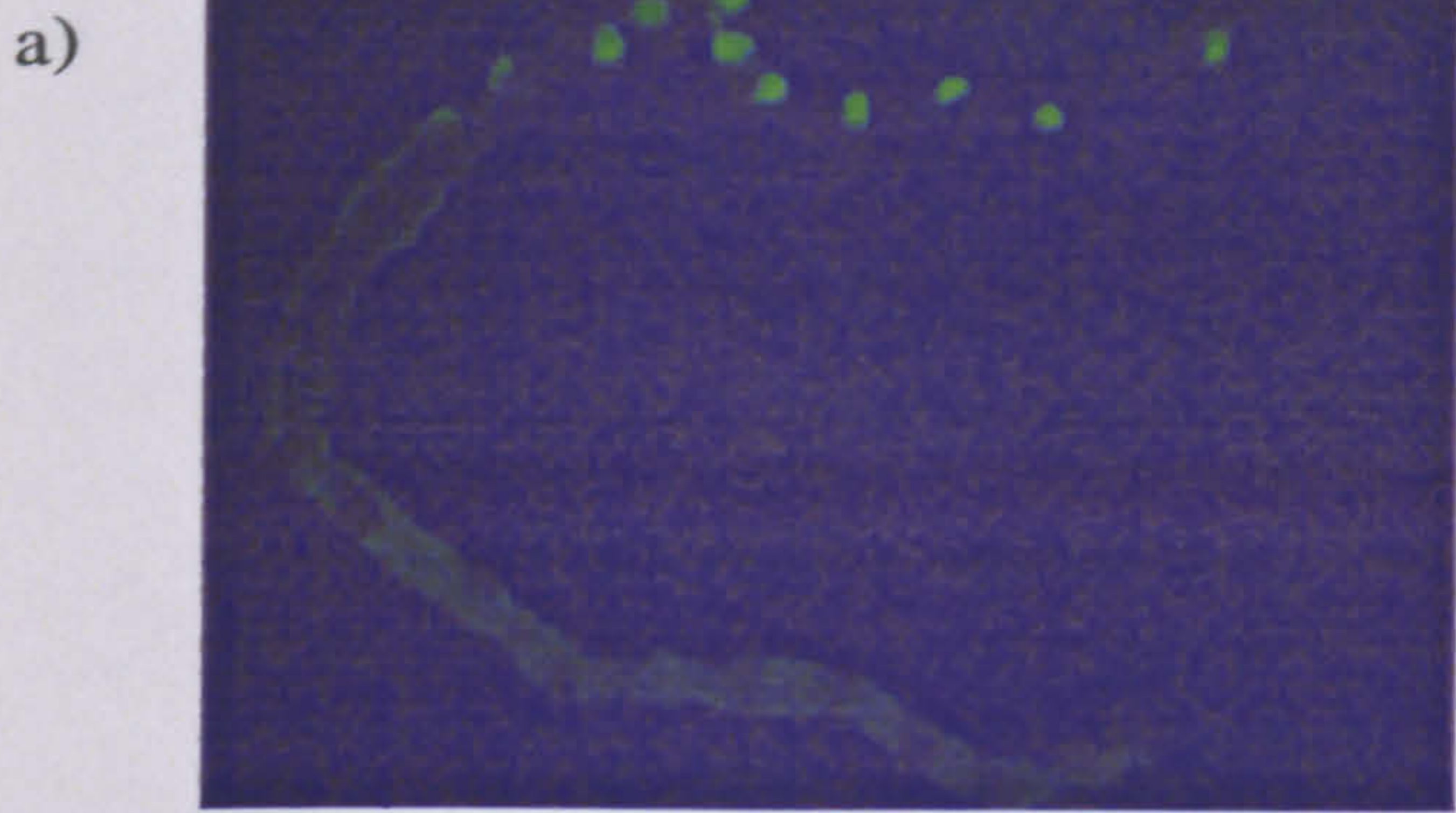


Figure 3.20. Line c649. Larval (3rd instar) GAL4-directed GFP expression patterns of Malpighian tubules by epifluorescence.

a) GAL4 expression in anterior tubules is confined to the stellate cells of the initial and transitional segments (almost complementary to line c710), and is not detectable in the stellate cells of the main segment, or anywhere in the posterior tubules.

b) The confocal picture of the same expression pattern.

c,d) Some of the stellate cells equivalent to bar-shaped ones in the distal part of the anterior Malpighian tubules.

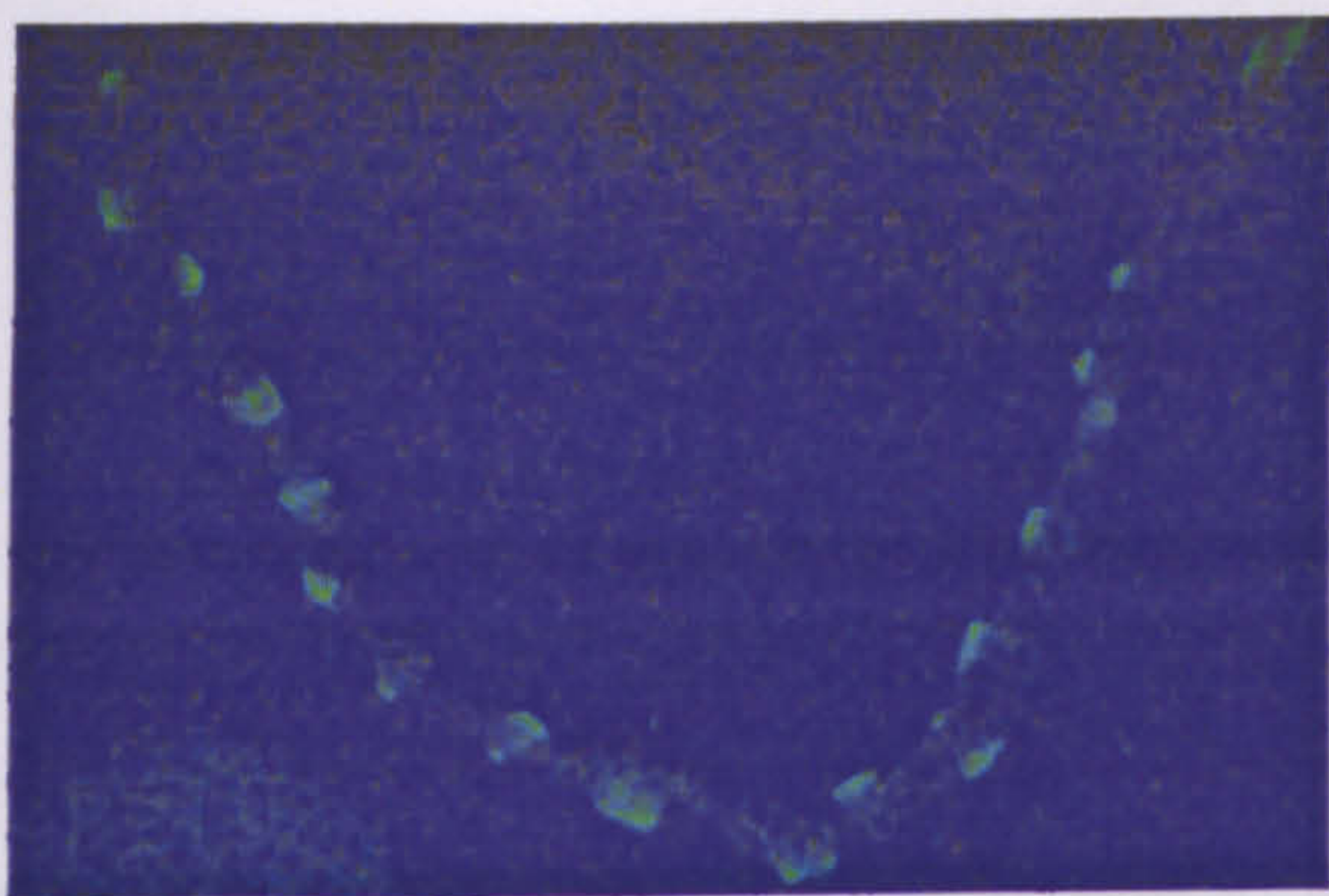
e) A single stellate cell from the initial-transitional area of anterior Malpighian tubules at higher magnification.

f) GAL4 expression pattern in the posterior Malpighian tubules. As can be seen, there is no detectable expression in the stellate cells.

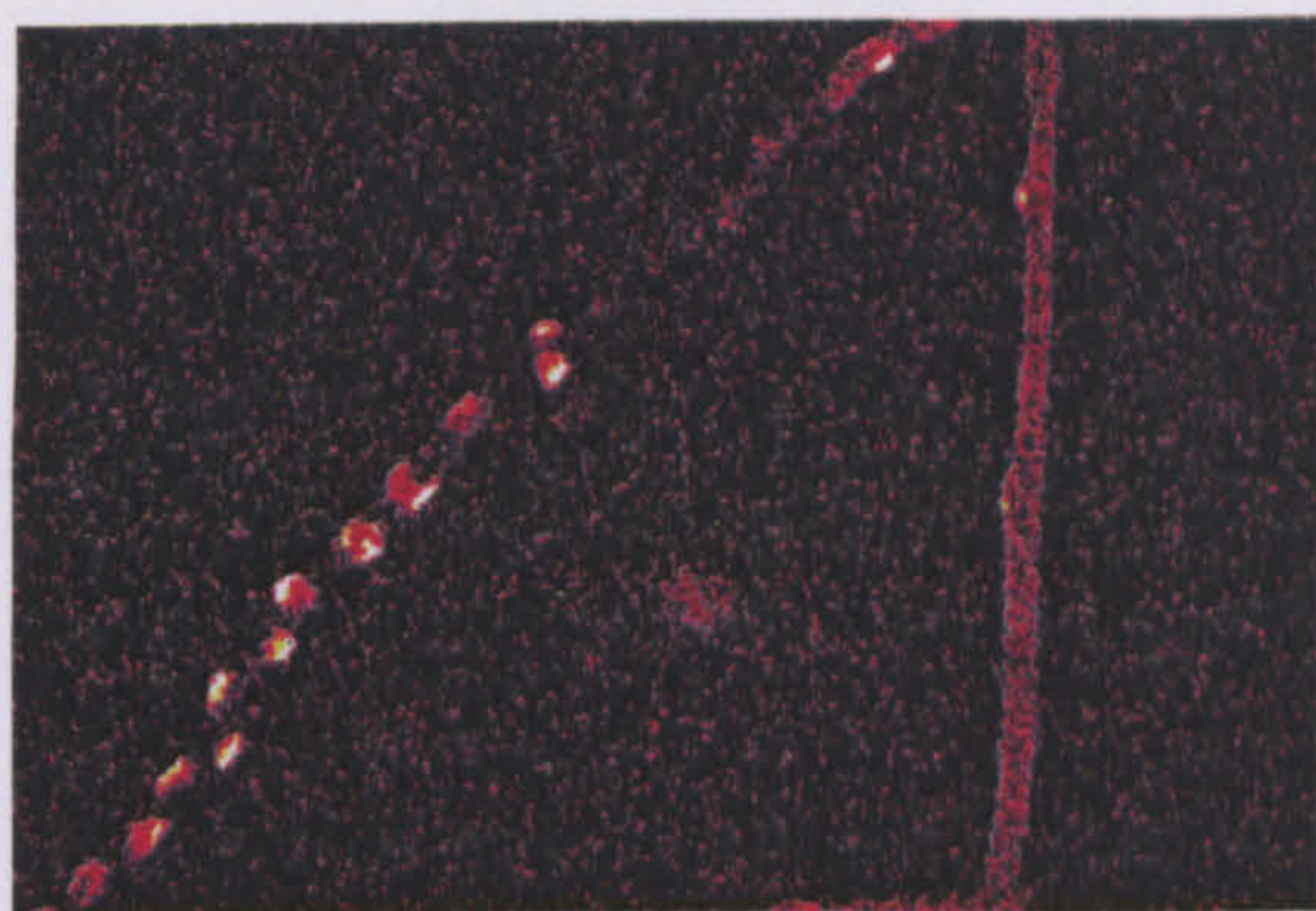
g) GAL4 expression simultaneously can be detected in the upper part of the ureter.

h) The higher magnification of the same expression pattern.

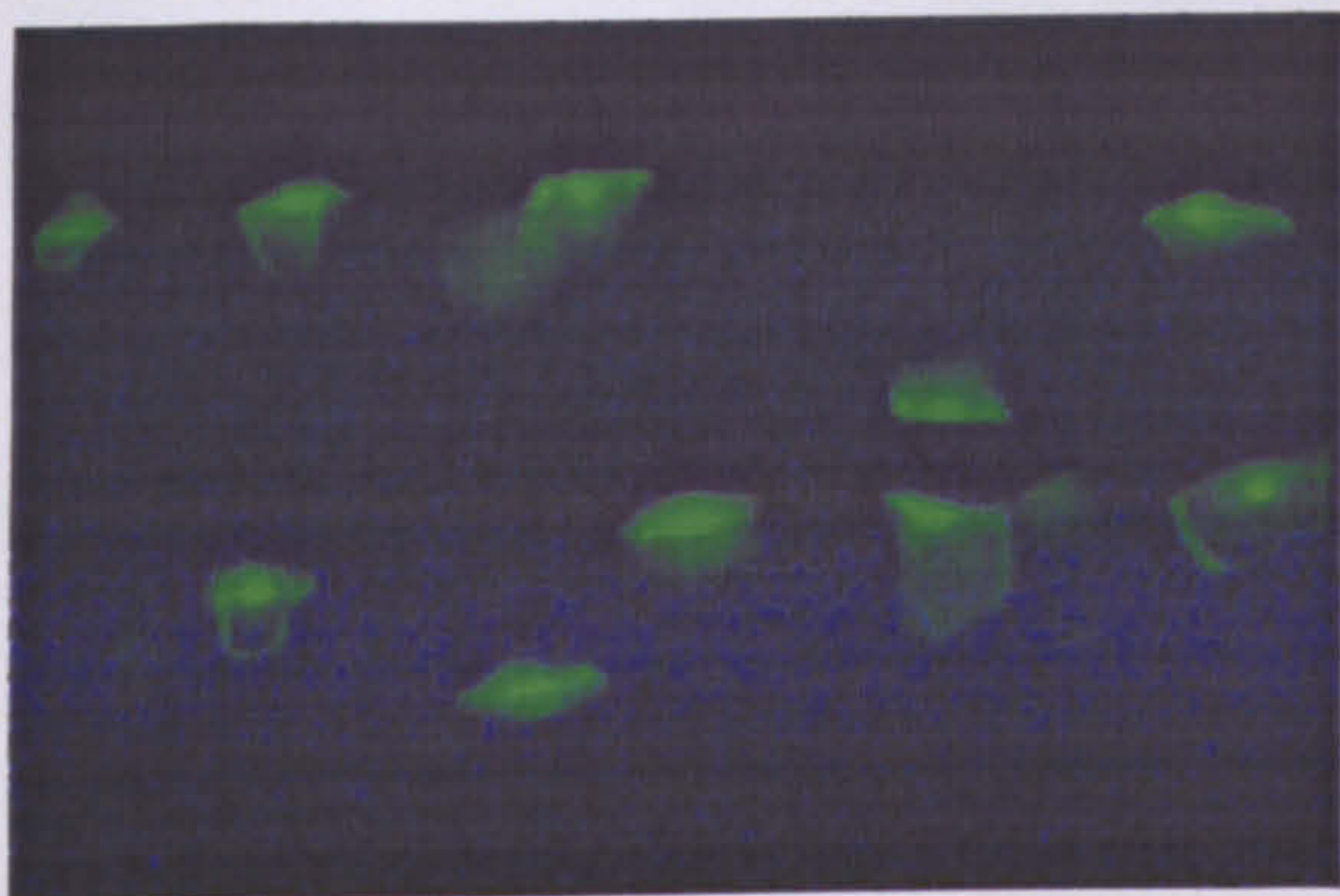
a)



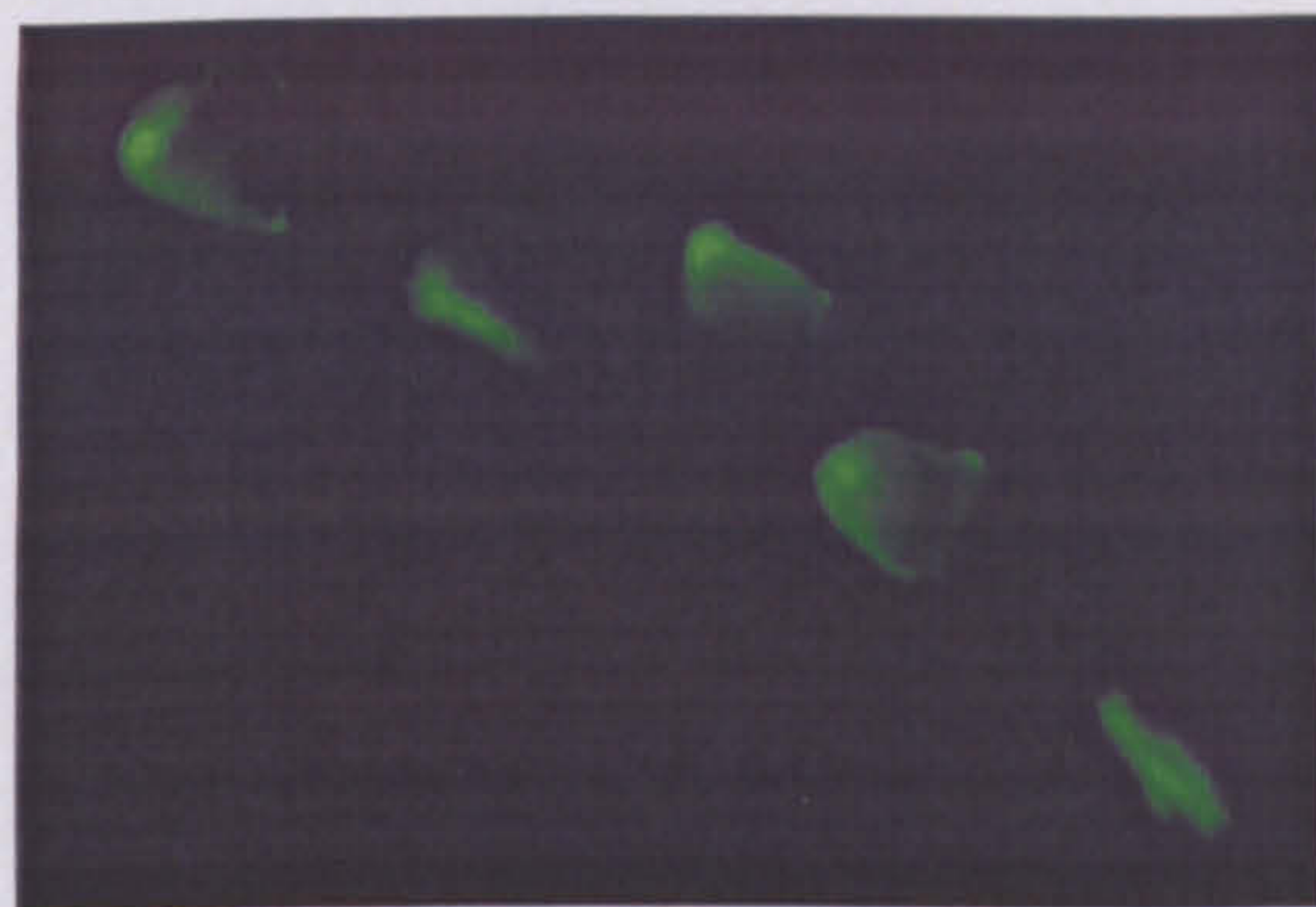
b)



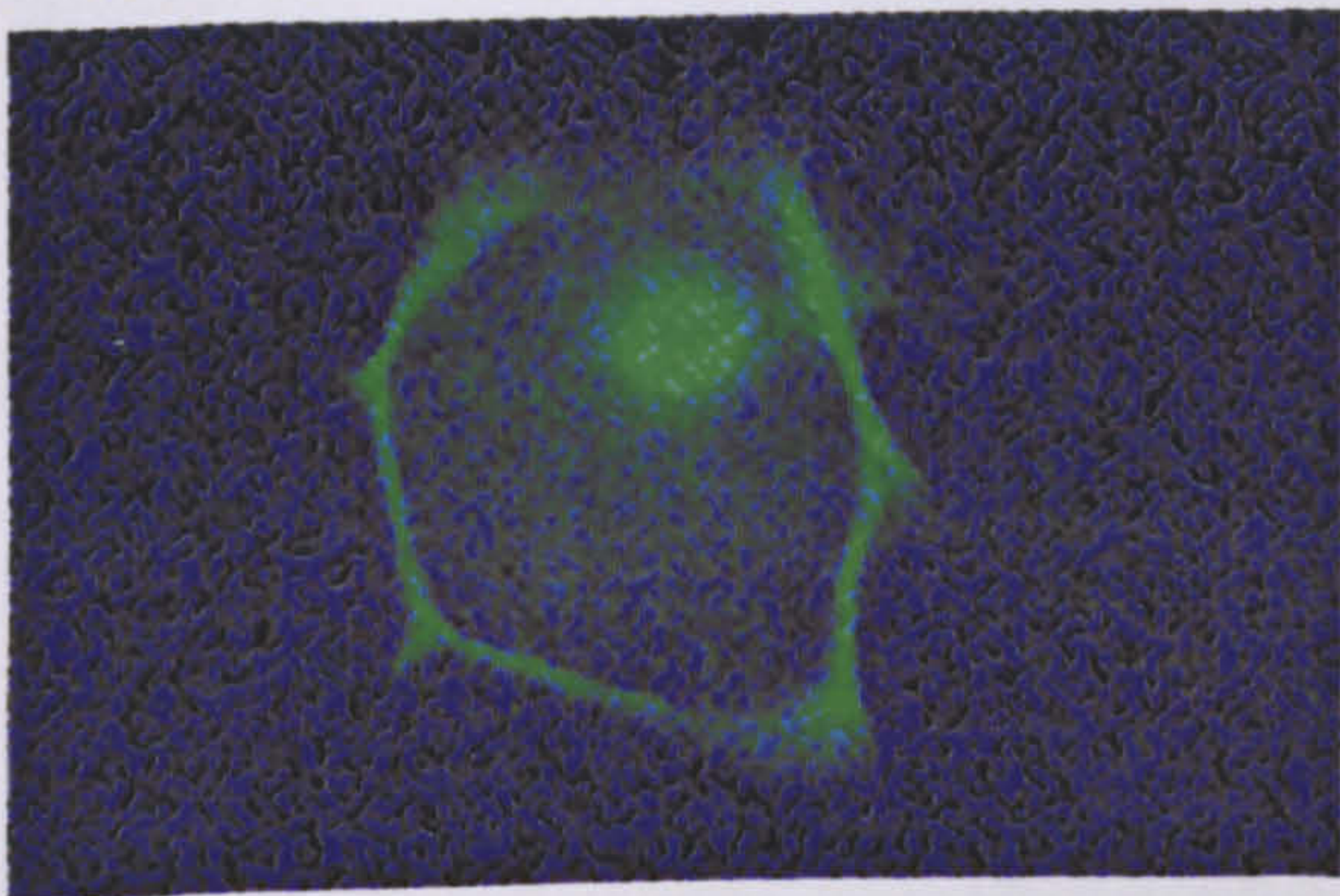
c)



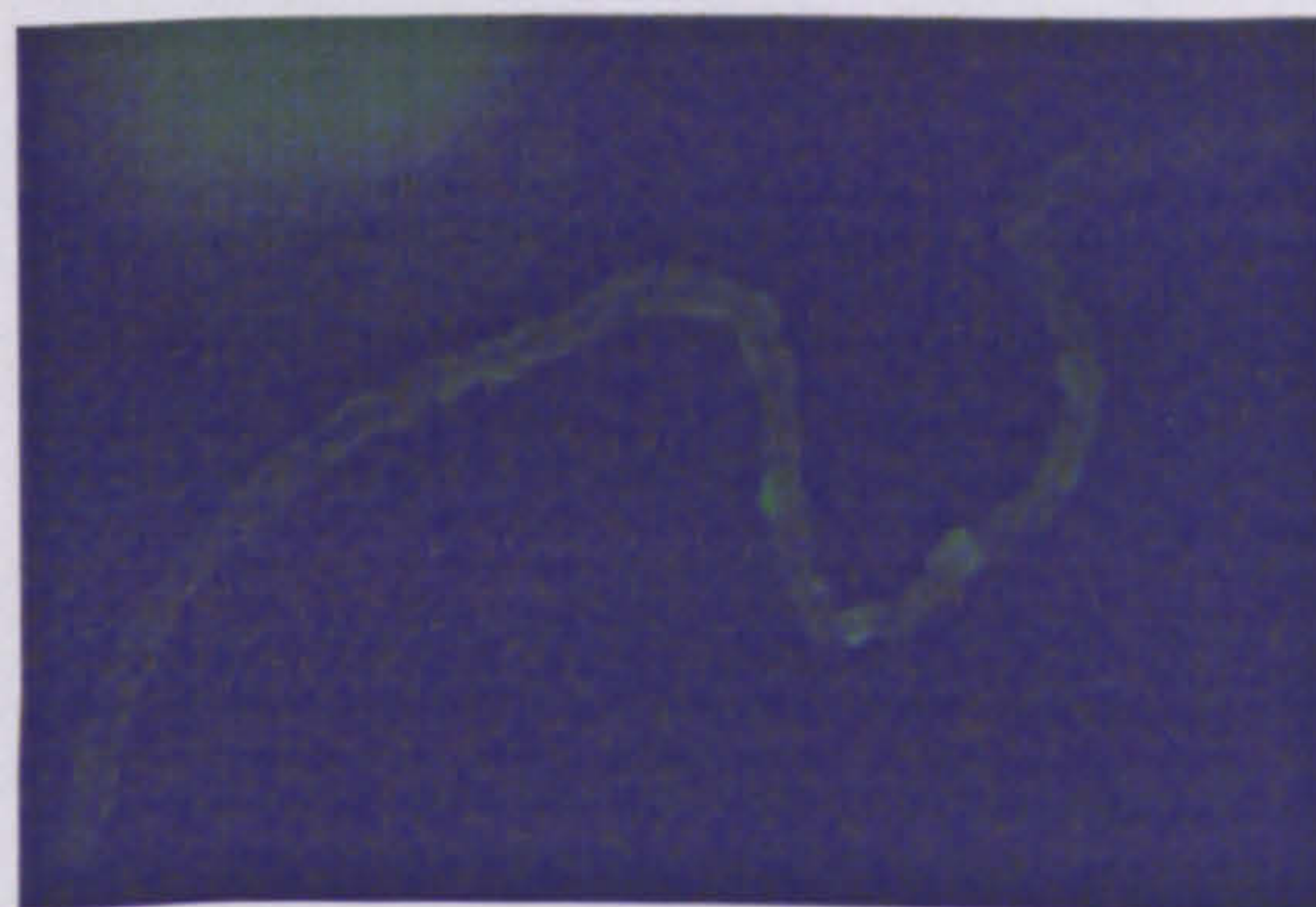
d)



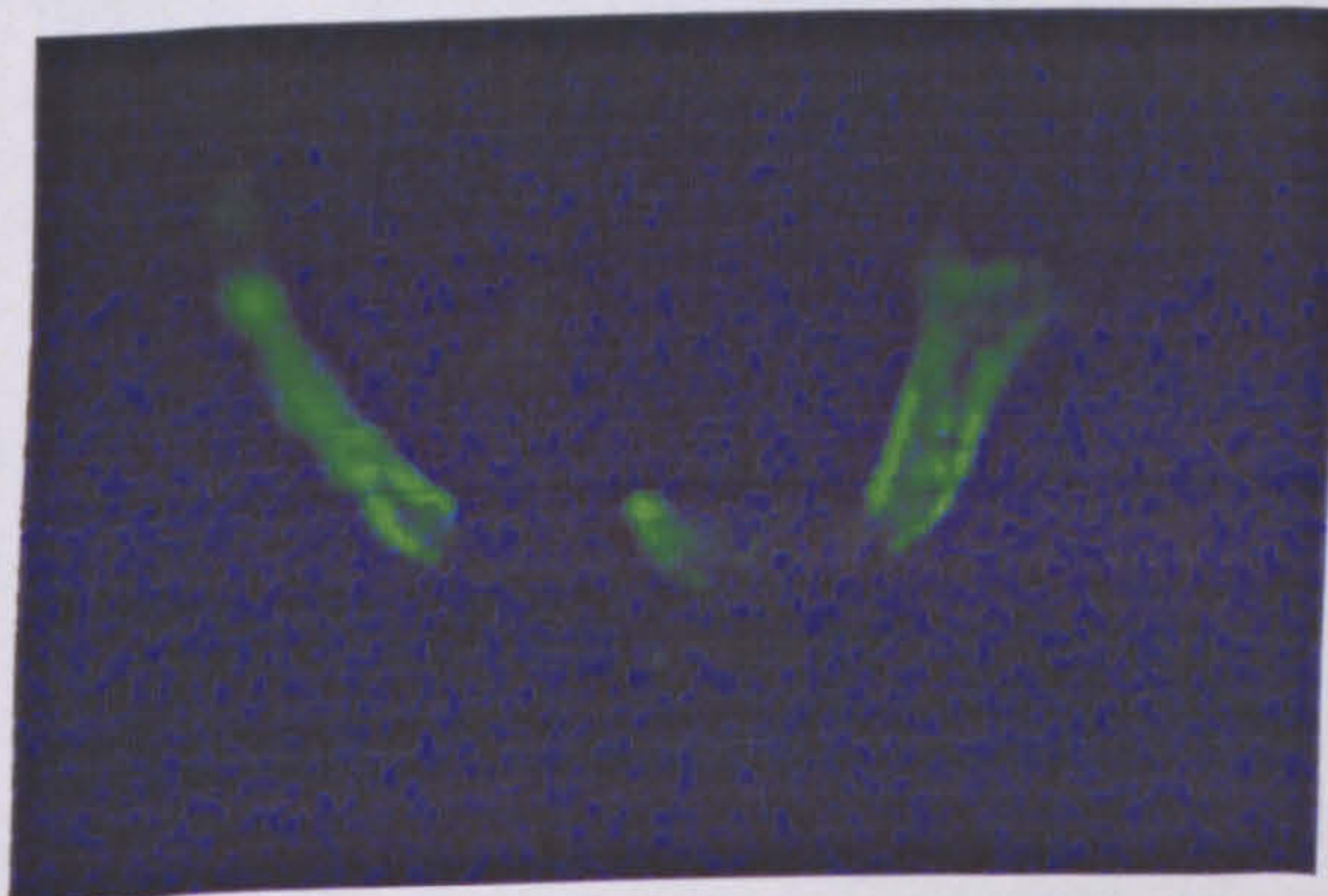
e)



f)



g)



h)

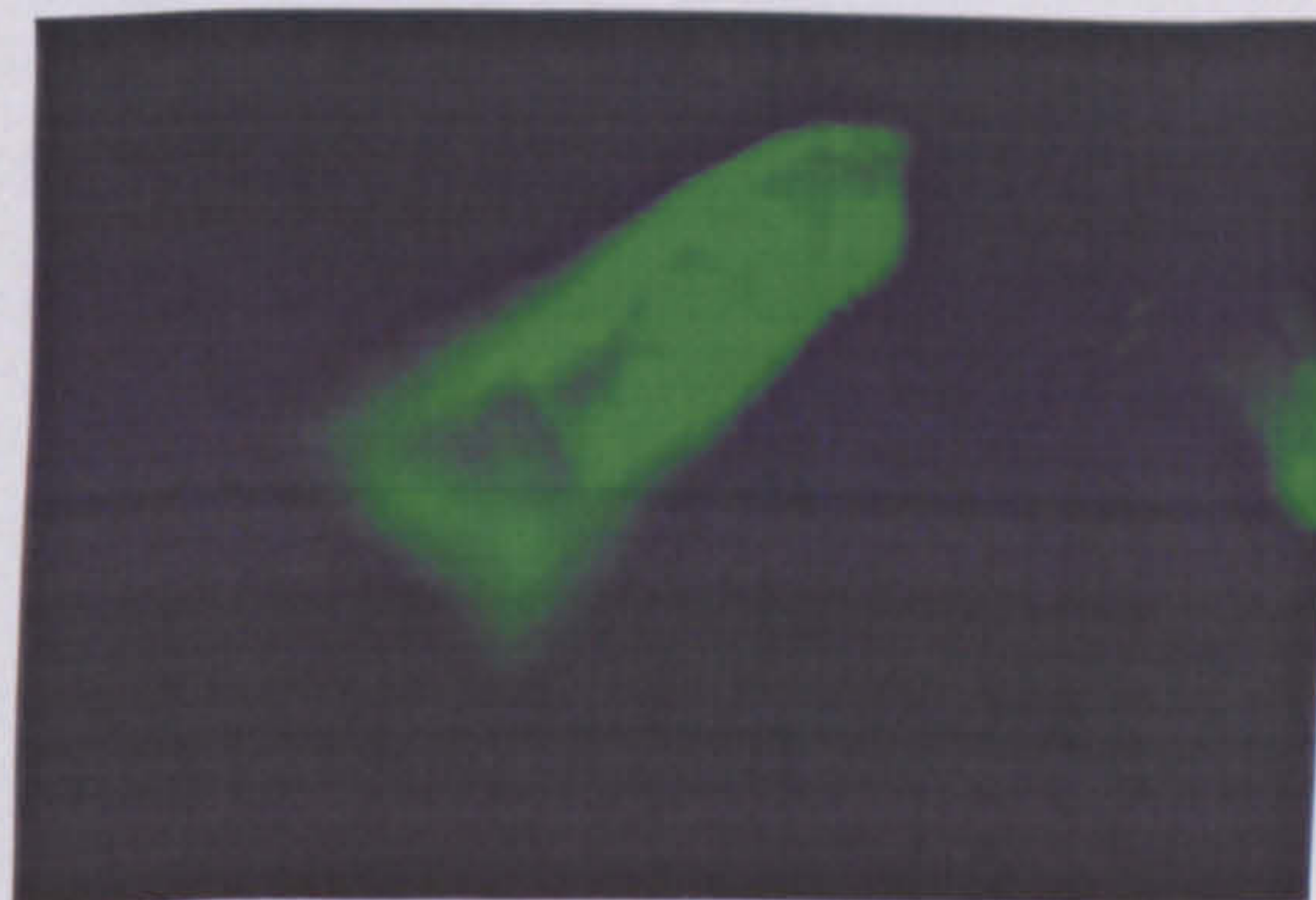
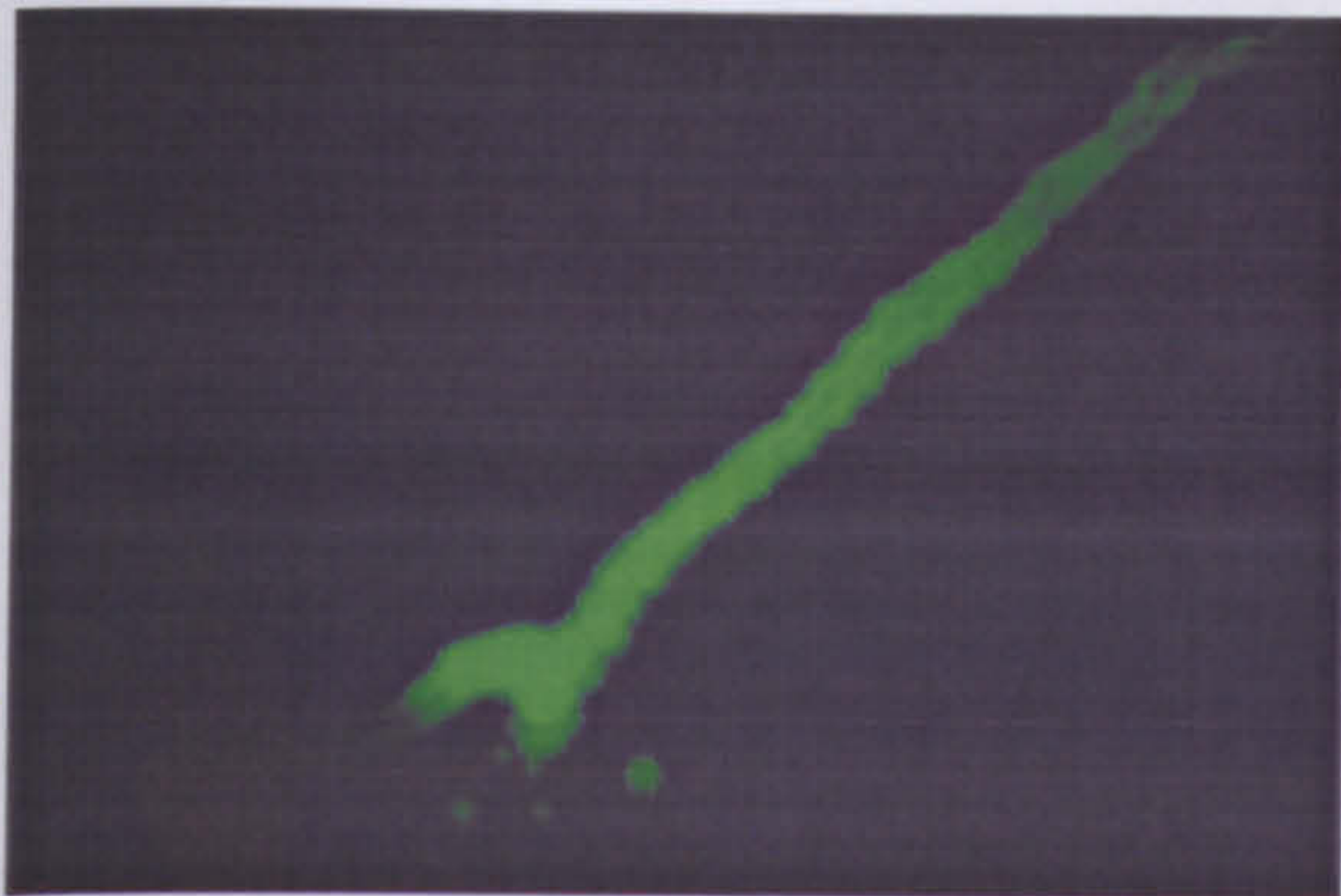


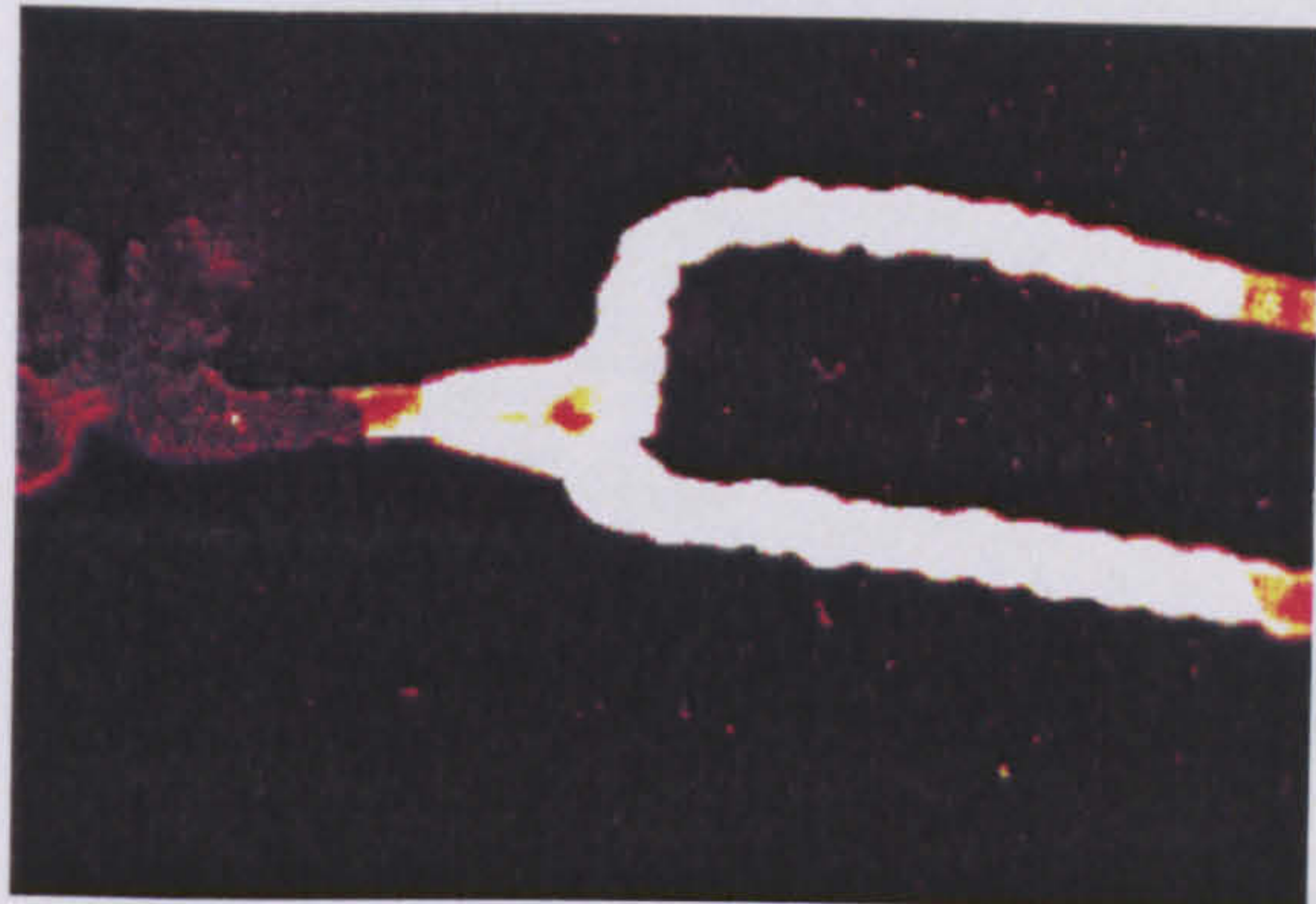
Figure 3.21. Line c507. GAL4-directed GFP expression reveals another comparative developmental diversity of expression pattern .

- a) GAL4 expression in the lower tubule and upper ureter obtained by epifluorescence in the third instar larva.
 - b) The same expression pattern obtained by confocal microscopy.
 - c) The distinction of upper and lower ureter boundary revealed by line c507.
 - d) In pupa, GFP is expressed also in the lower tubule and upper ureter not in the lower ureter with the confocal picture.
 - e) In adult the expression is extended to the lower ureter (the confocal image) in addition to the lower tubule and upper part of ureter.
 - f) The same expression pattern obtained under epiflorescence.
 - g) In larva (3rd instar) the distal part of the tubule (probably initial and transitional segments) is also labelled by GAL4 expression except the bar-shaped cells of these segments.
 - h) A higher magnification of the distal part: stellate cells of this part are not included in the expression.
-

a)



b)



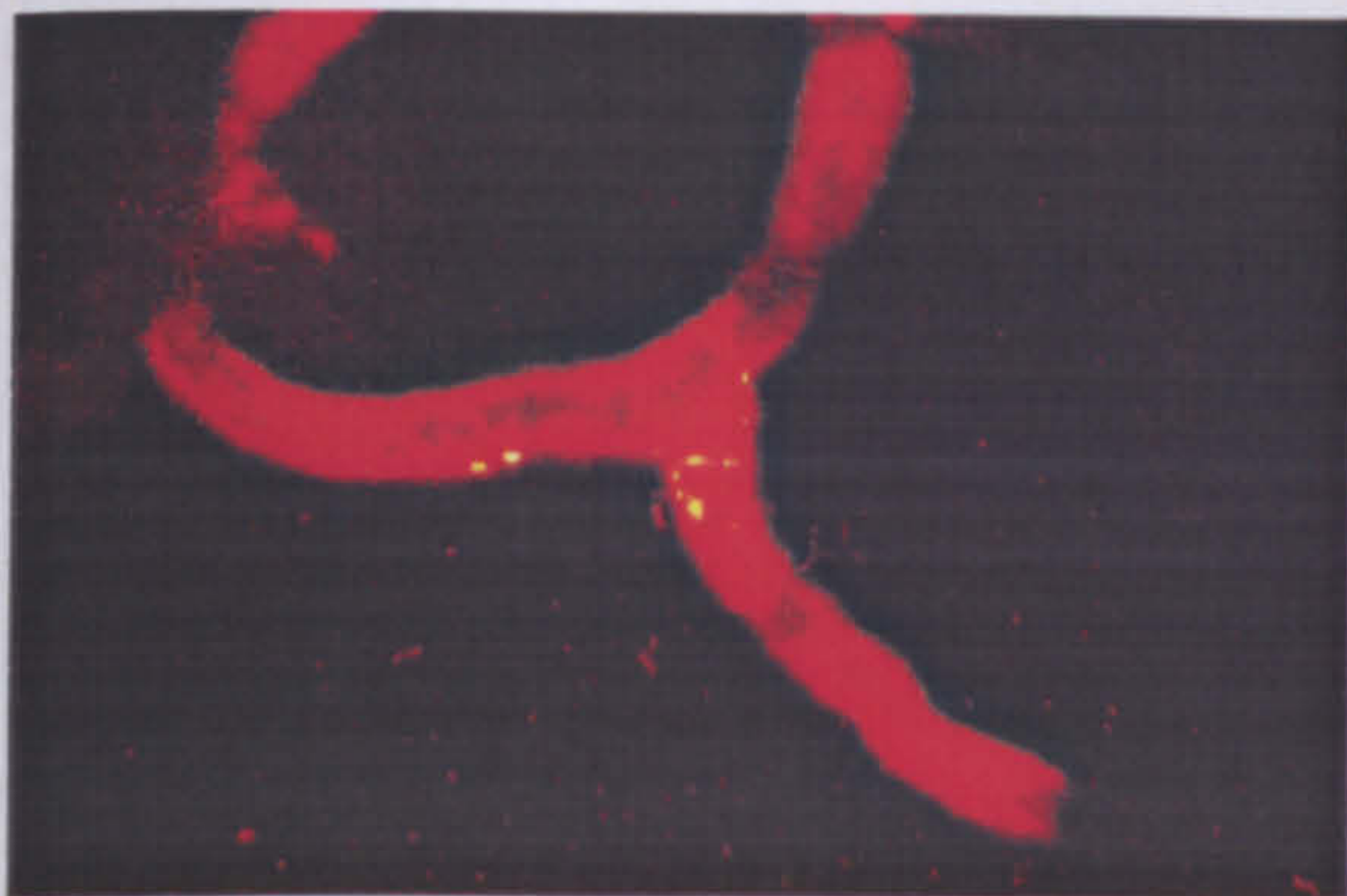
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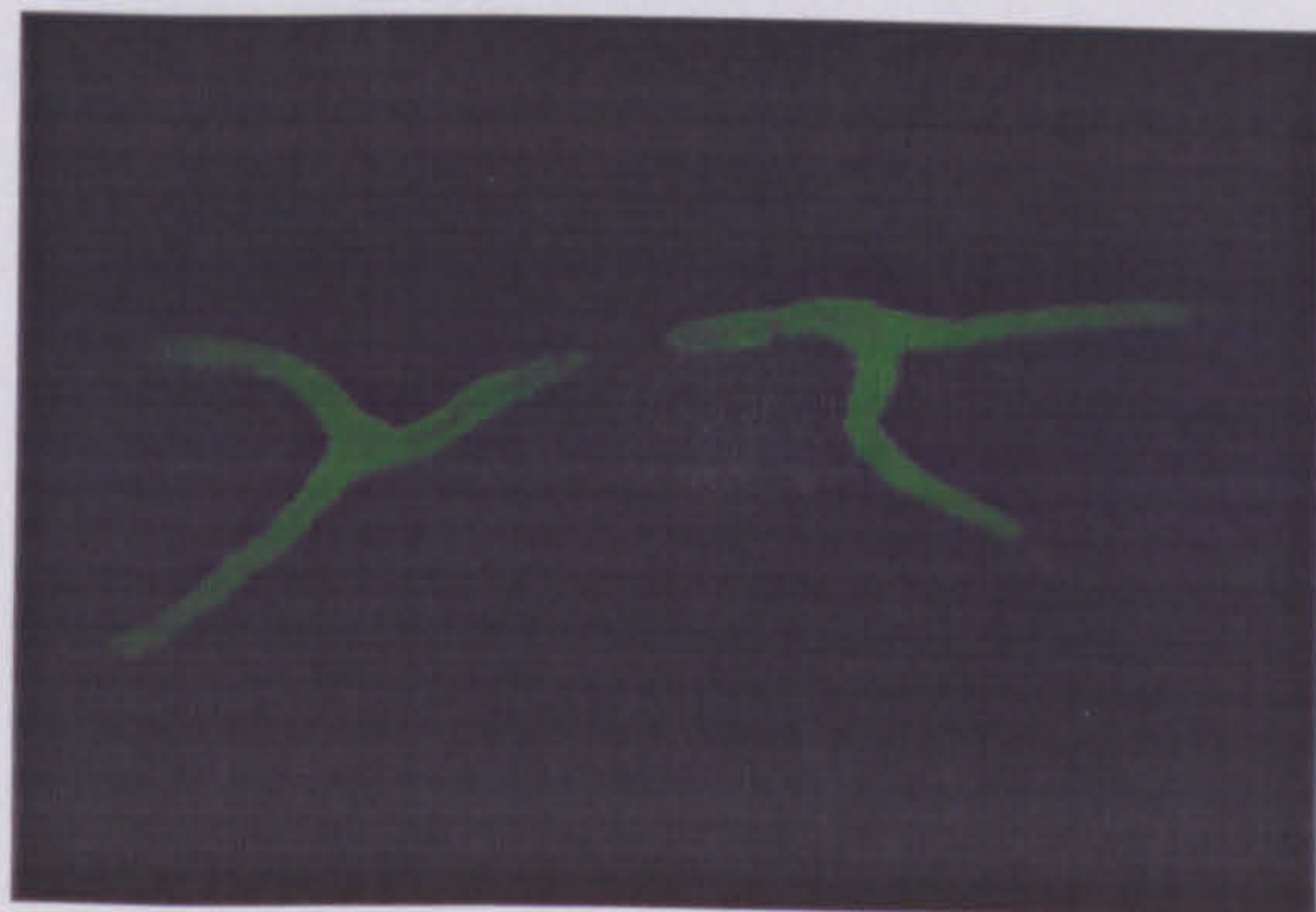
d)



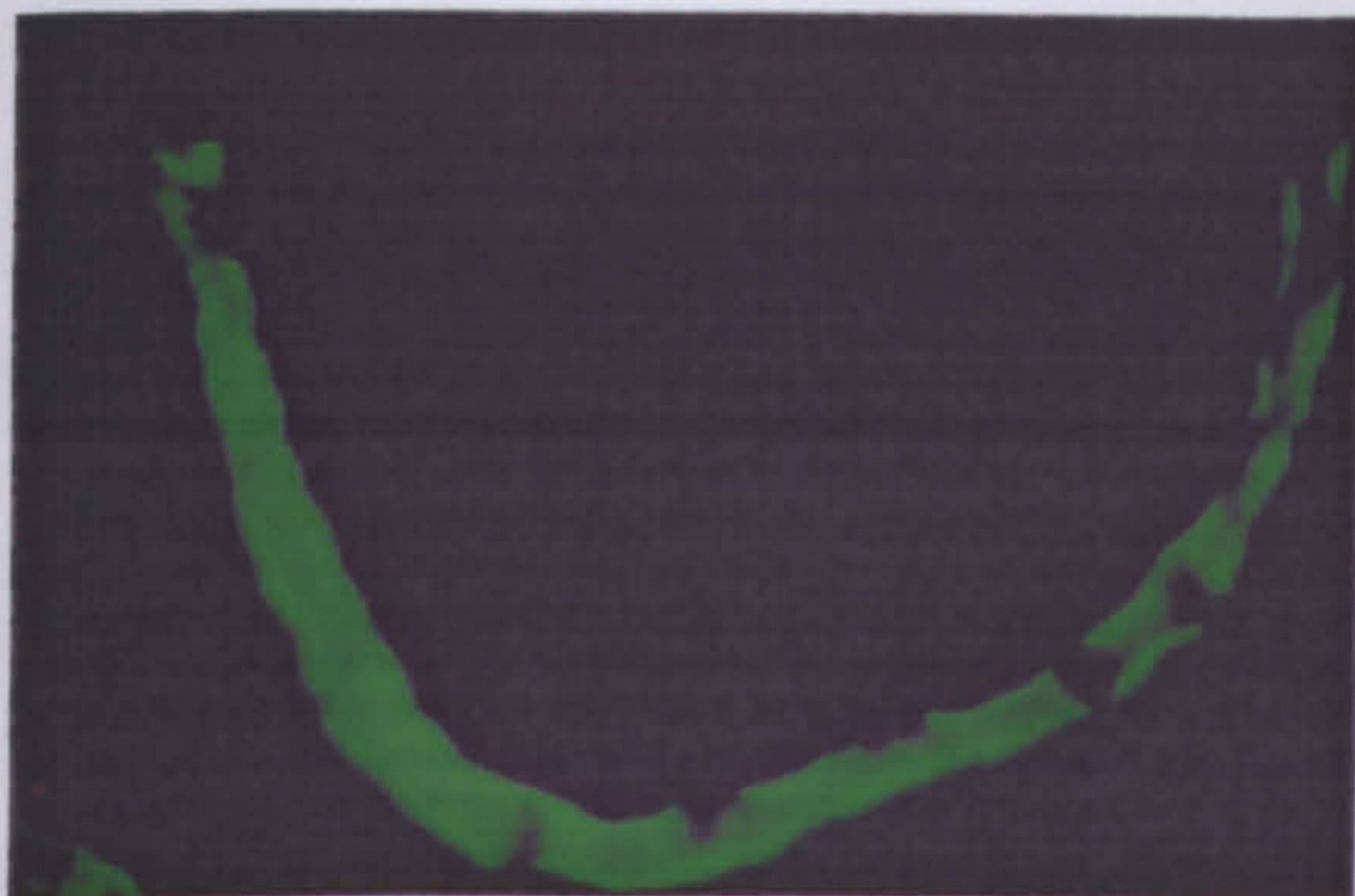
e)



f)



g)



h)

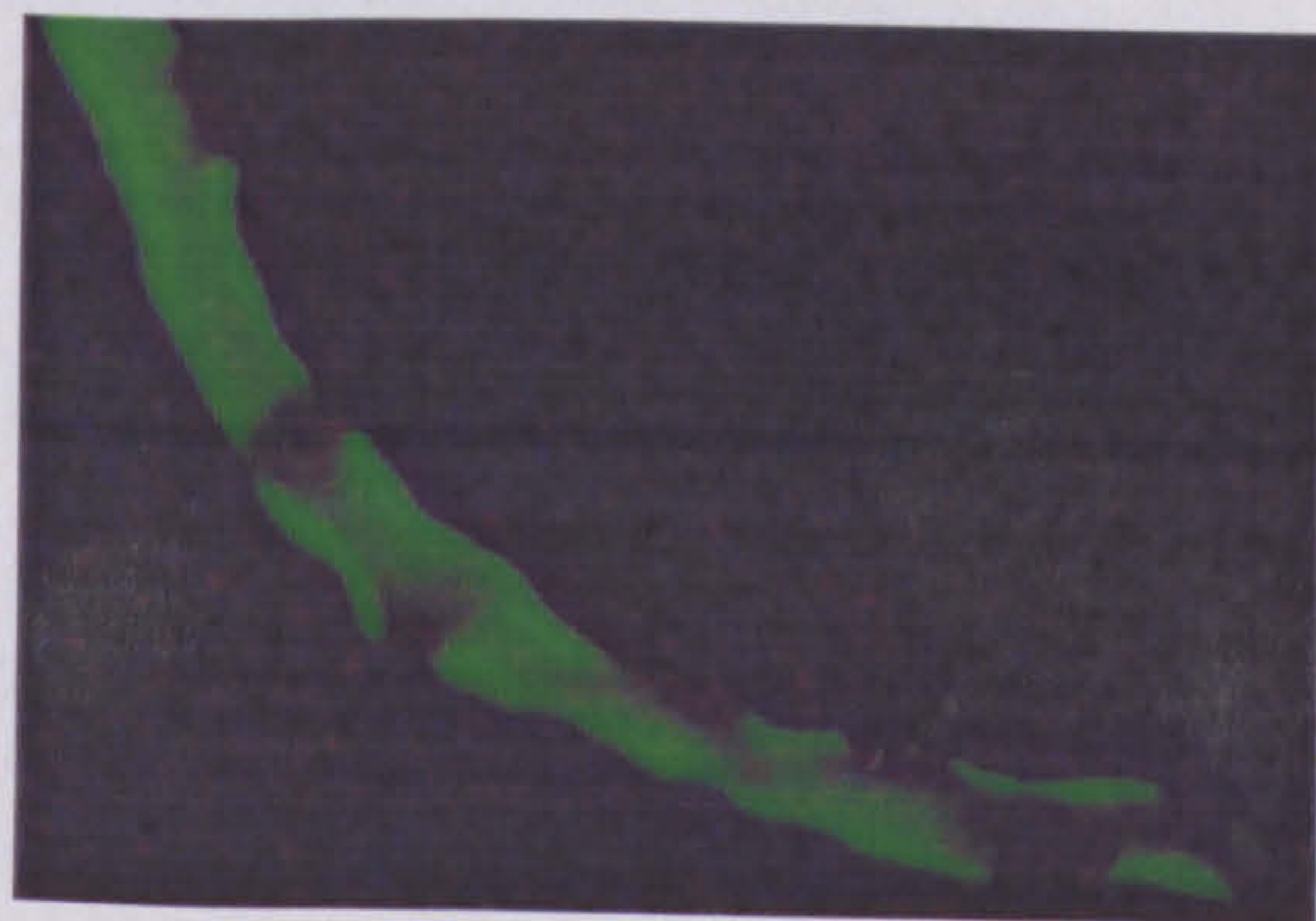
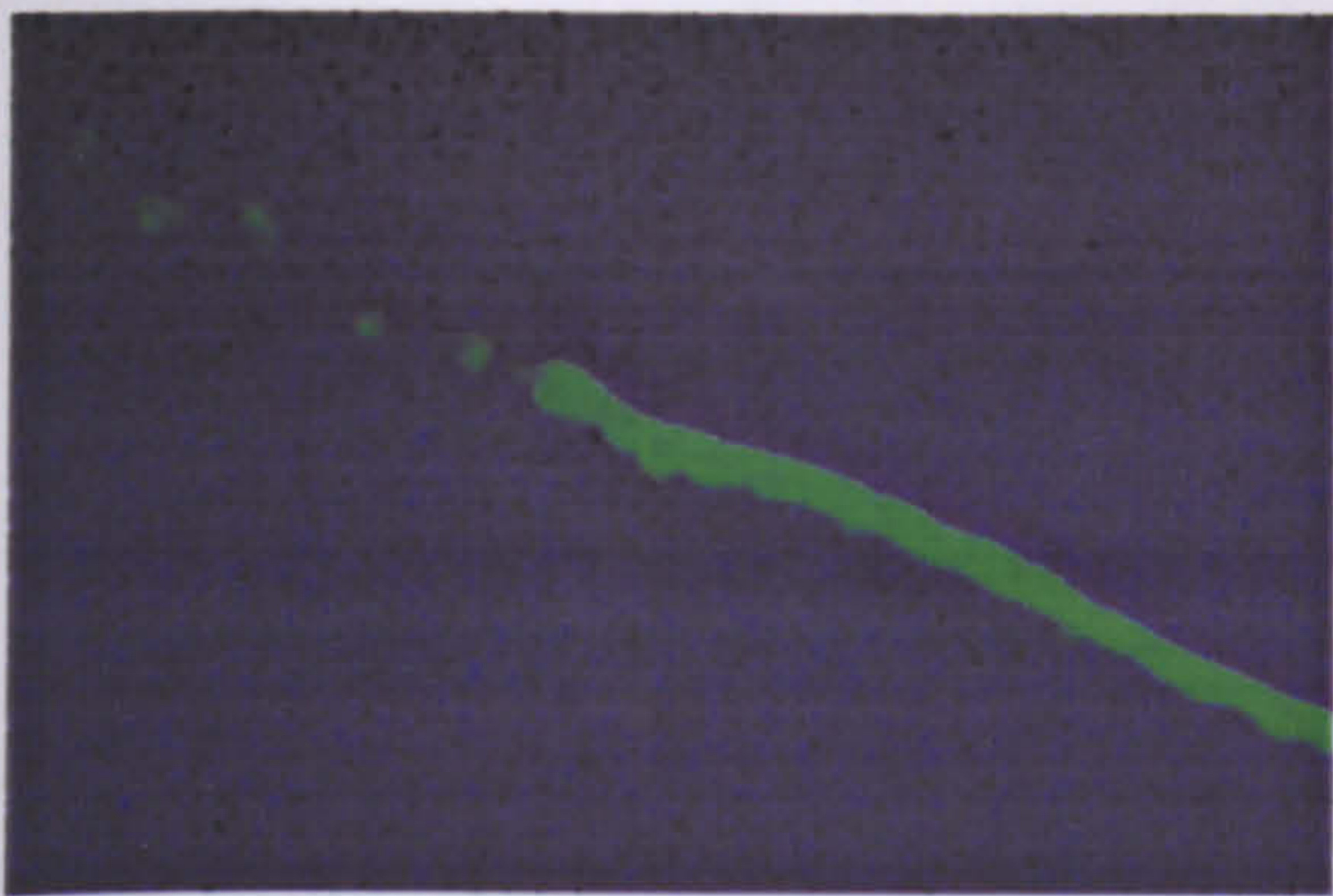


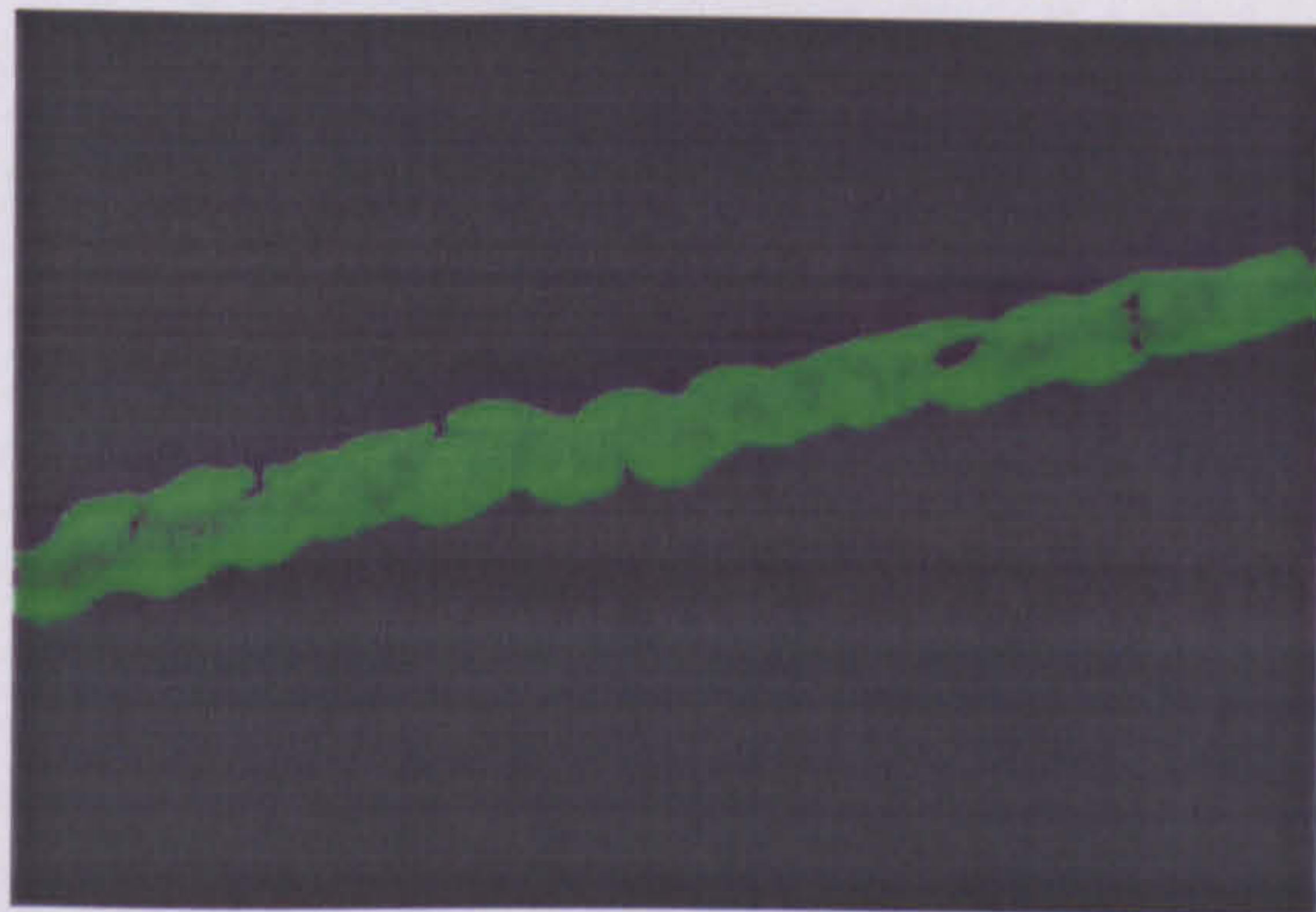
Figure 3.22. Line C42. Larval GAL4-directed GFP expression patterns of Malpighian tubules by epifluorescence.

- a) GAL4 is expressed in the principal cells except in the distal part (initial and transitional segments) and lower ureter and stellate cells equivalent to bar-shaped ones in the distal part, but not the main segment.
 - b) The non-staining stellate cells in the main segment at higher magnification.
 - c) The expression pattern detectable in the stellate cells in the distal part.
 - d) Four bar-shaped cells from the initial-transitional area of anterior Malpighian tubules at higher magnification.
 - e) The expression pattern in the posterior Malpighian tubules.
 - f) It turns out that the expression is excluded in the stellate cells of this region.
 - g) The expression pattern of both ureters.
 - h) It is clear that GAL4 expression is only in the upper part of the ureter, but not in the lower ureter.
-

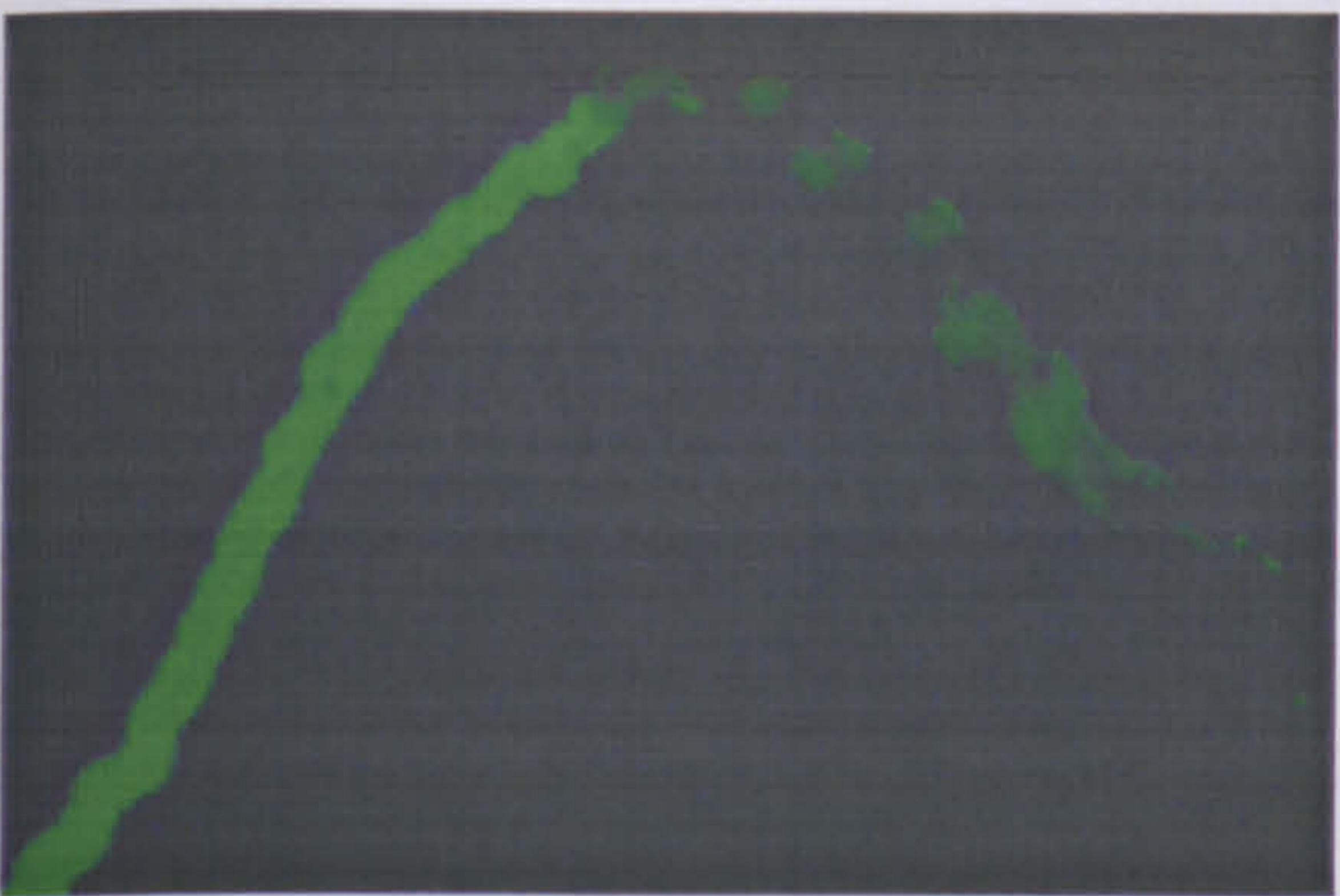
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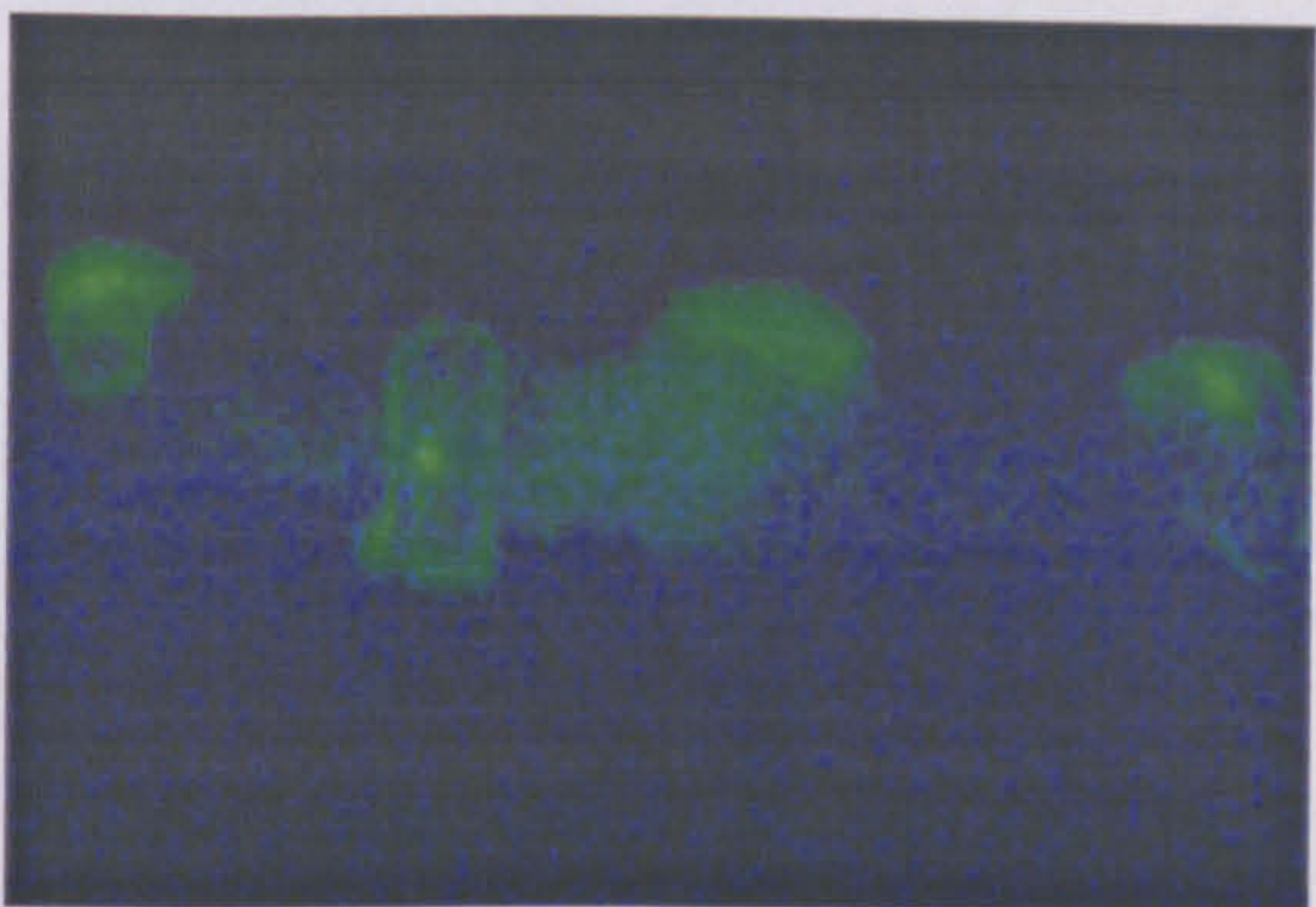
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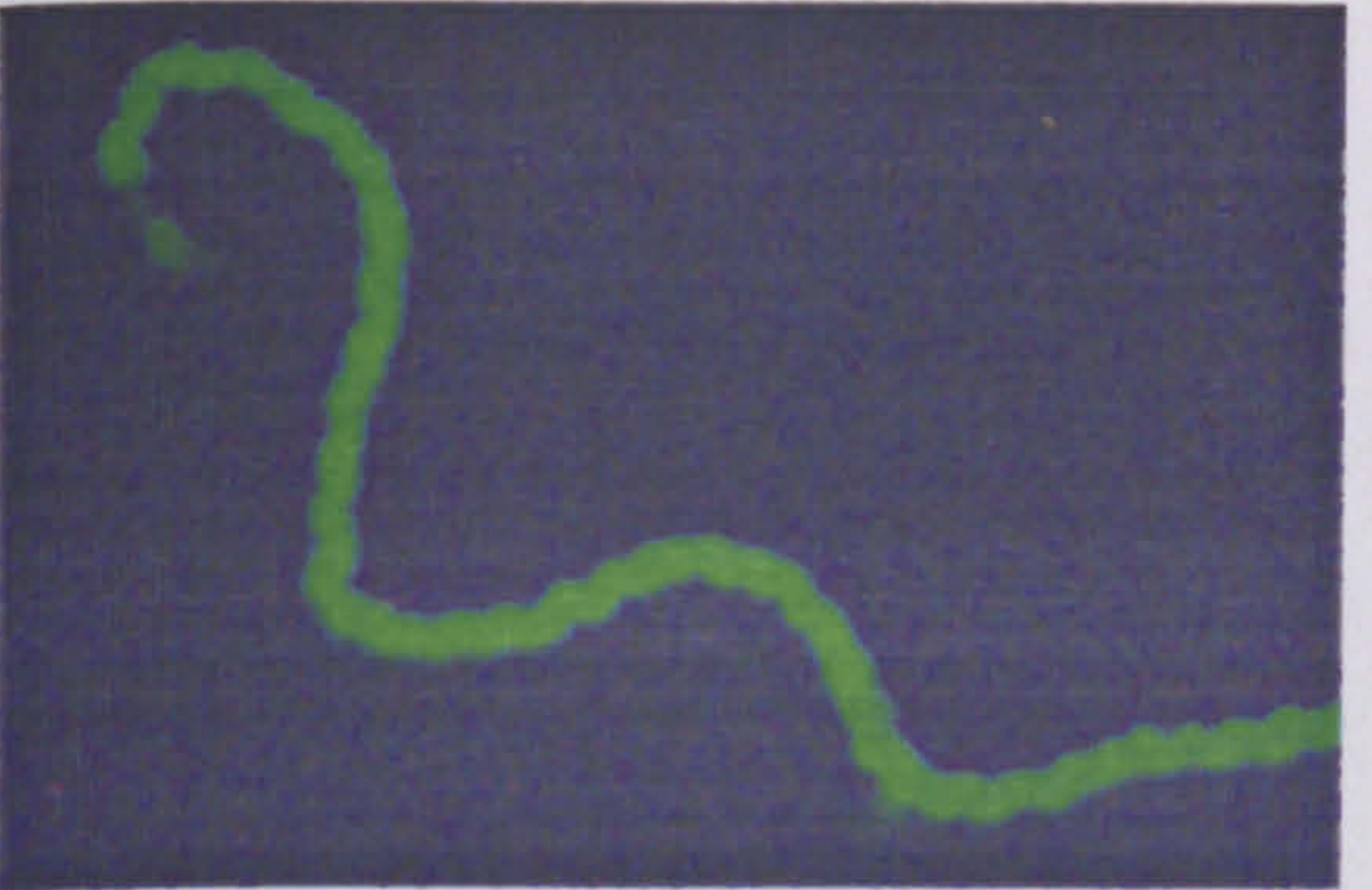
c)



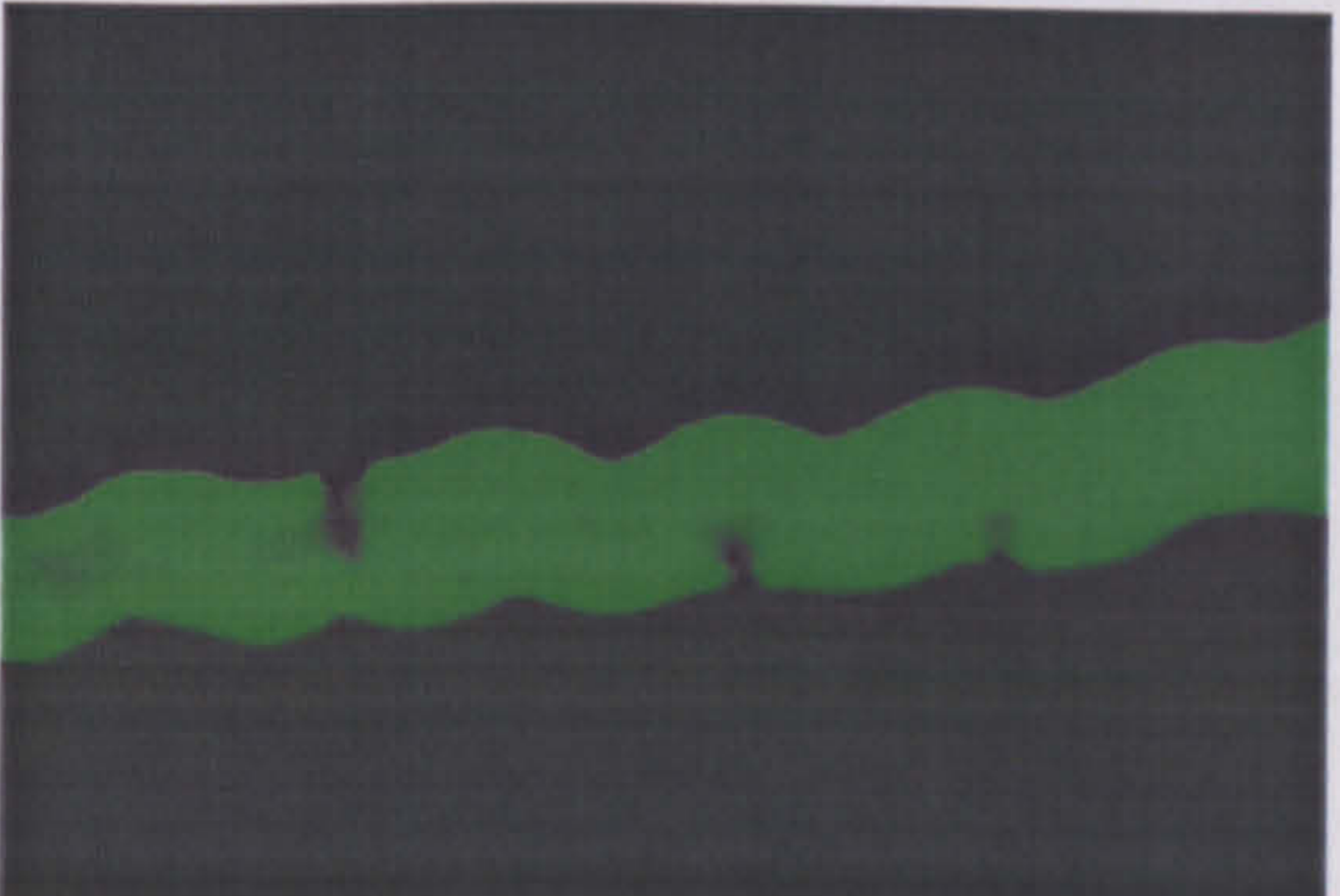
d)



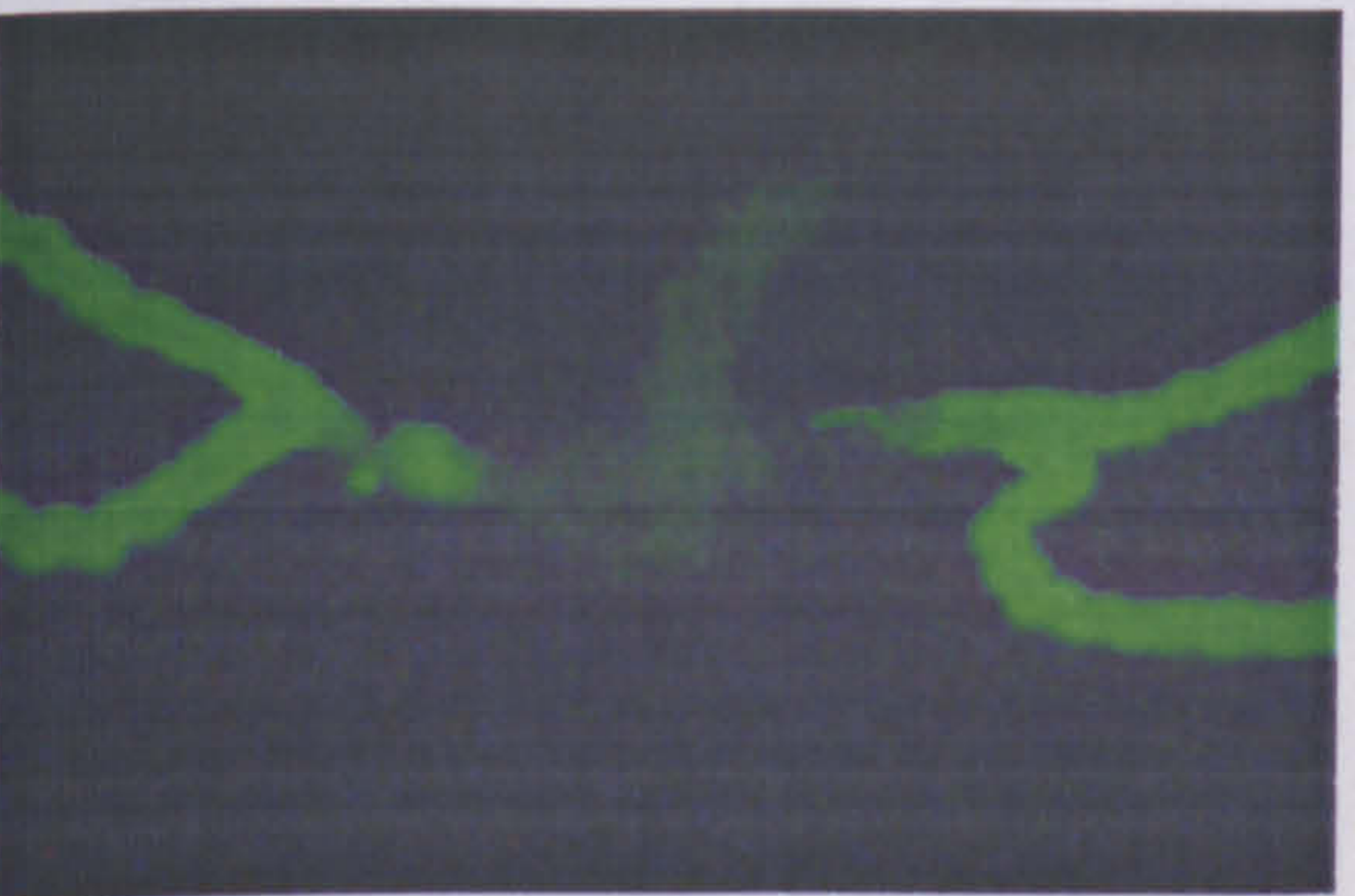
e)



f)



g)



h)

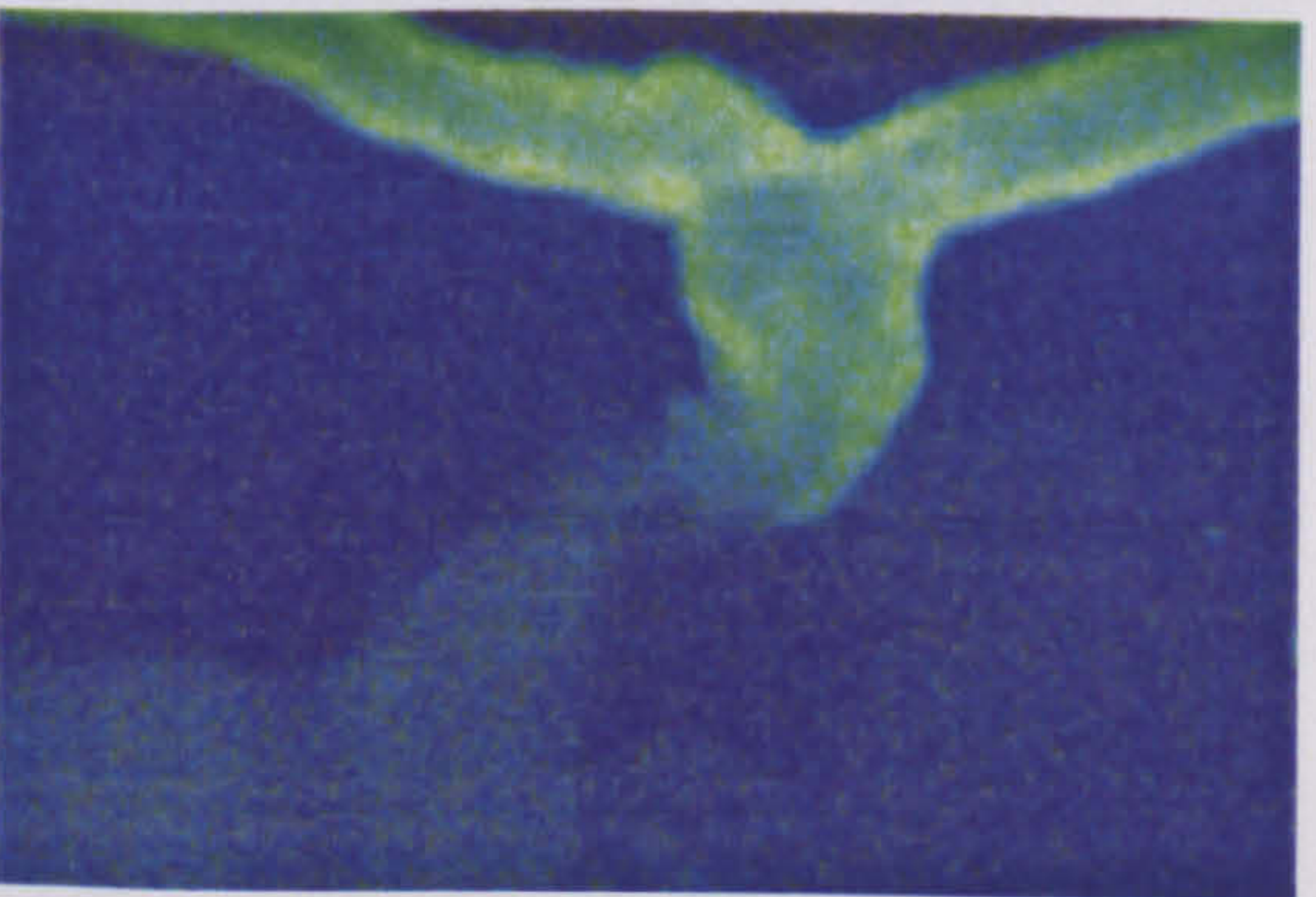
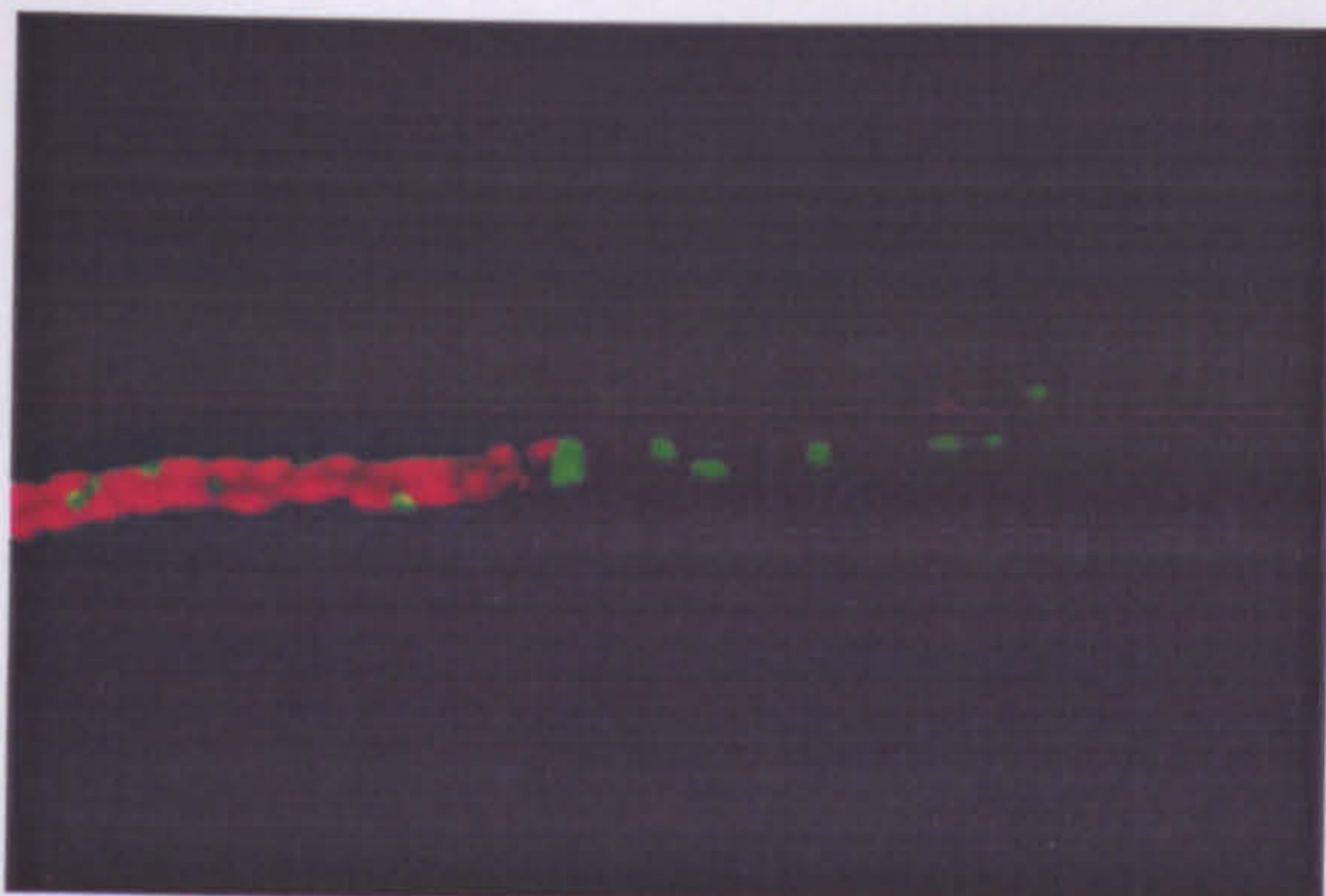


Figure 3.23. Functional distinction of two subtypes of stellate cells throughout developmental stages using GAL4 x UAS_G-GFP lines of C724.

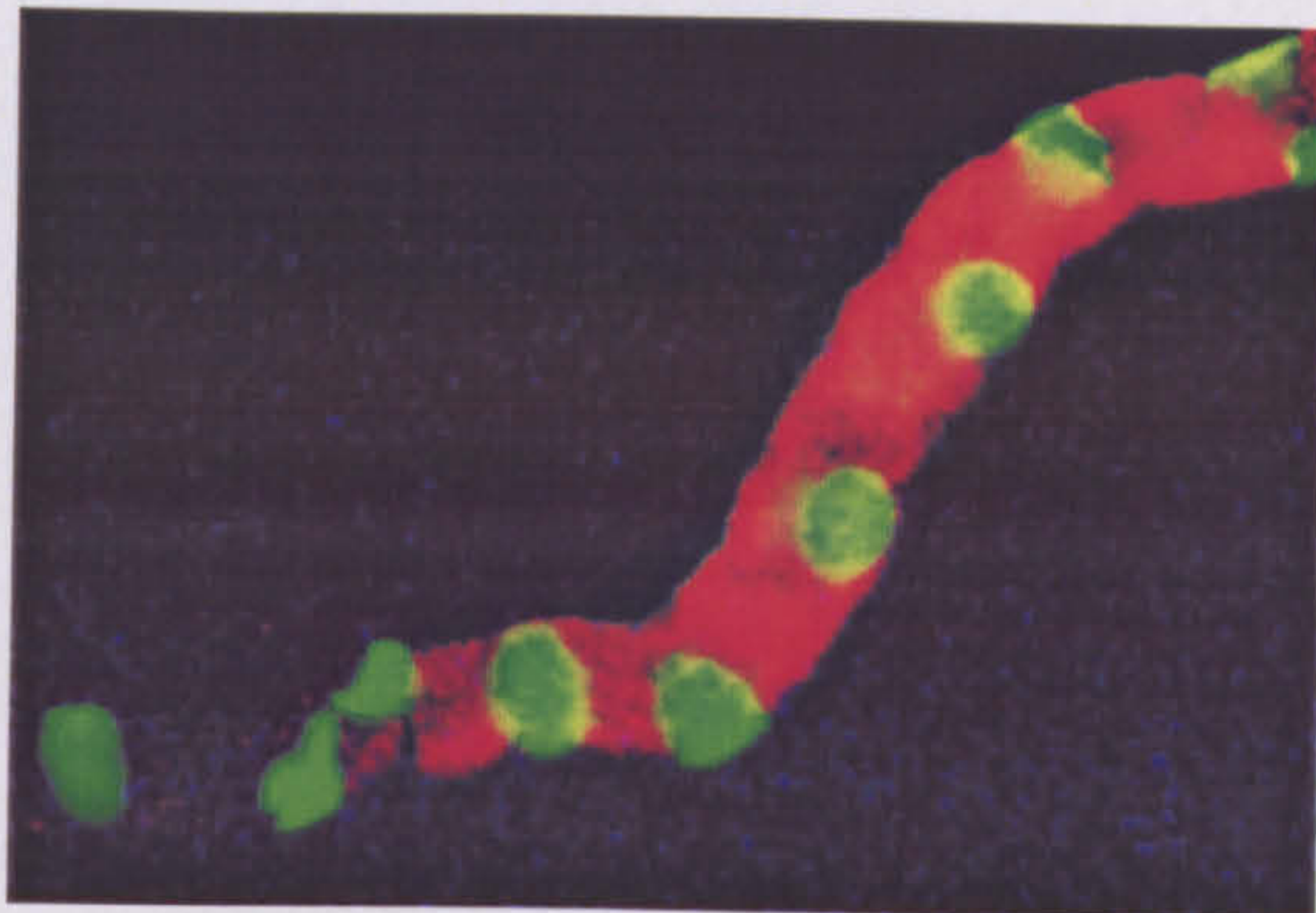
Repetition of transport assay with ethidium bromide (red) on the fluorescently labelled live tubules with GFP divides the regions with respect to the ion transport.

- a) The border of the transporting main segment and non-transporting transitional-initial segments of anterior tubule.
 - b) The same boundary probably overlaps with the border of two subtypes of stellate cells as close as can be resolved.
 - c) The fluorescently labelled live tubule with GFP from the third instar larva before the assay.
 - d) Following the assay it is clear that transport of ethidium bromide is to the main segment region, not the distal part, marked by bar-shaped counterparts of stellate cells.
 - e) The live labelled tubule from the pupa prior to the assay.
 - f) After the assay the same tubule showing two regions in accordance with solute transport.
 - g) The adult tubule labelled with GFP in the stellate cells before the assay.
 - h) The same tubule after the assay.
-

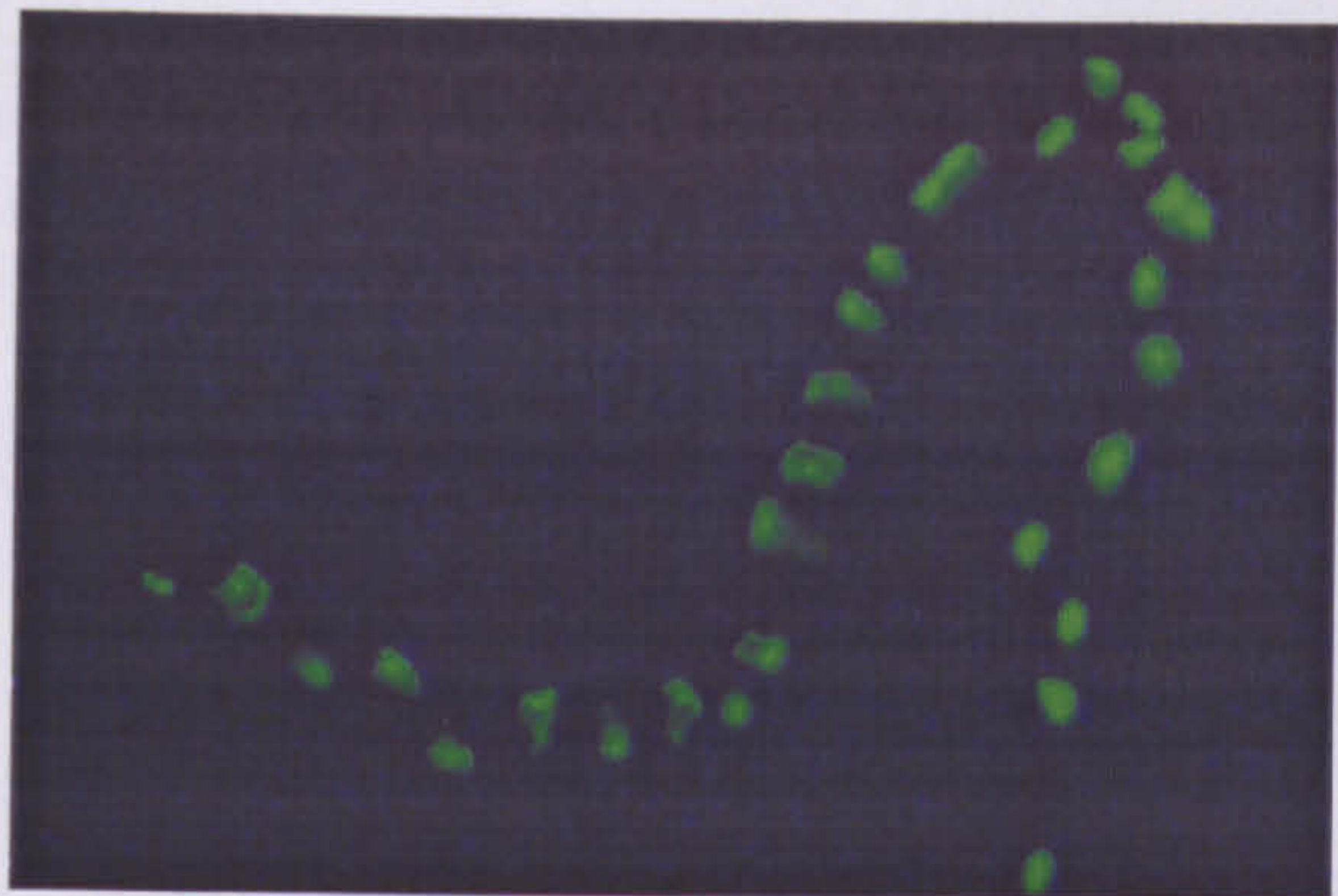
a)



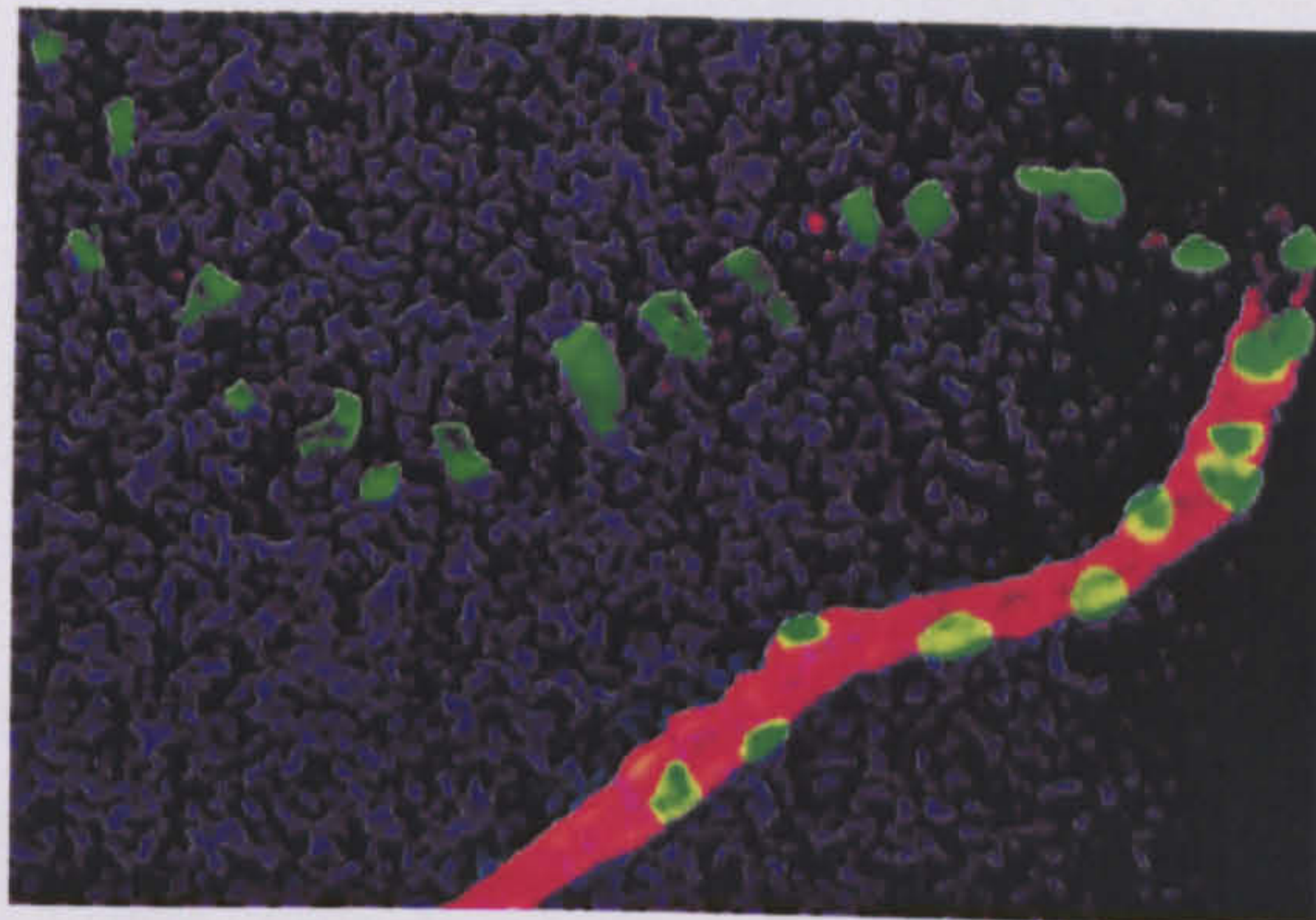
b)



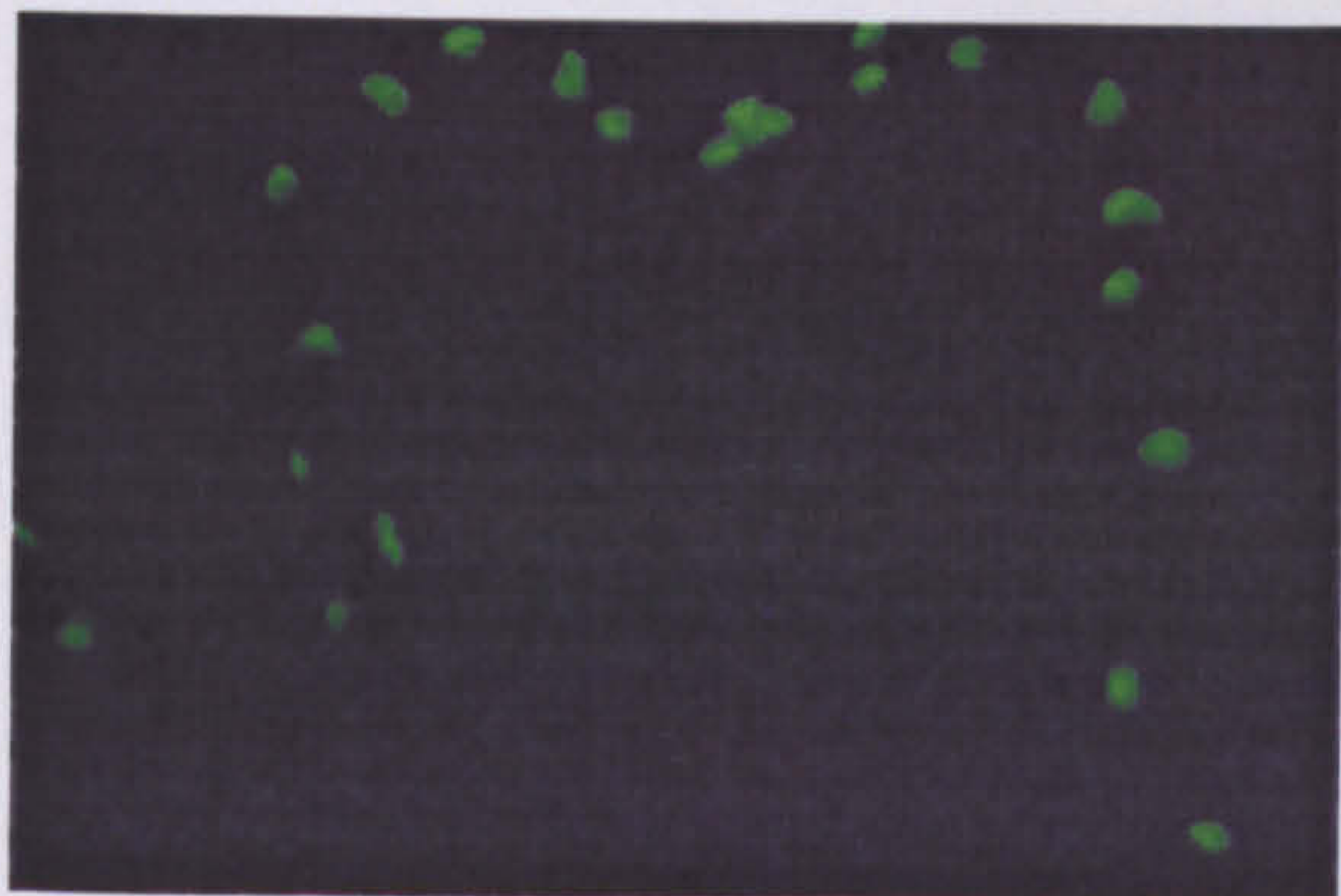
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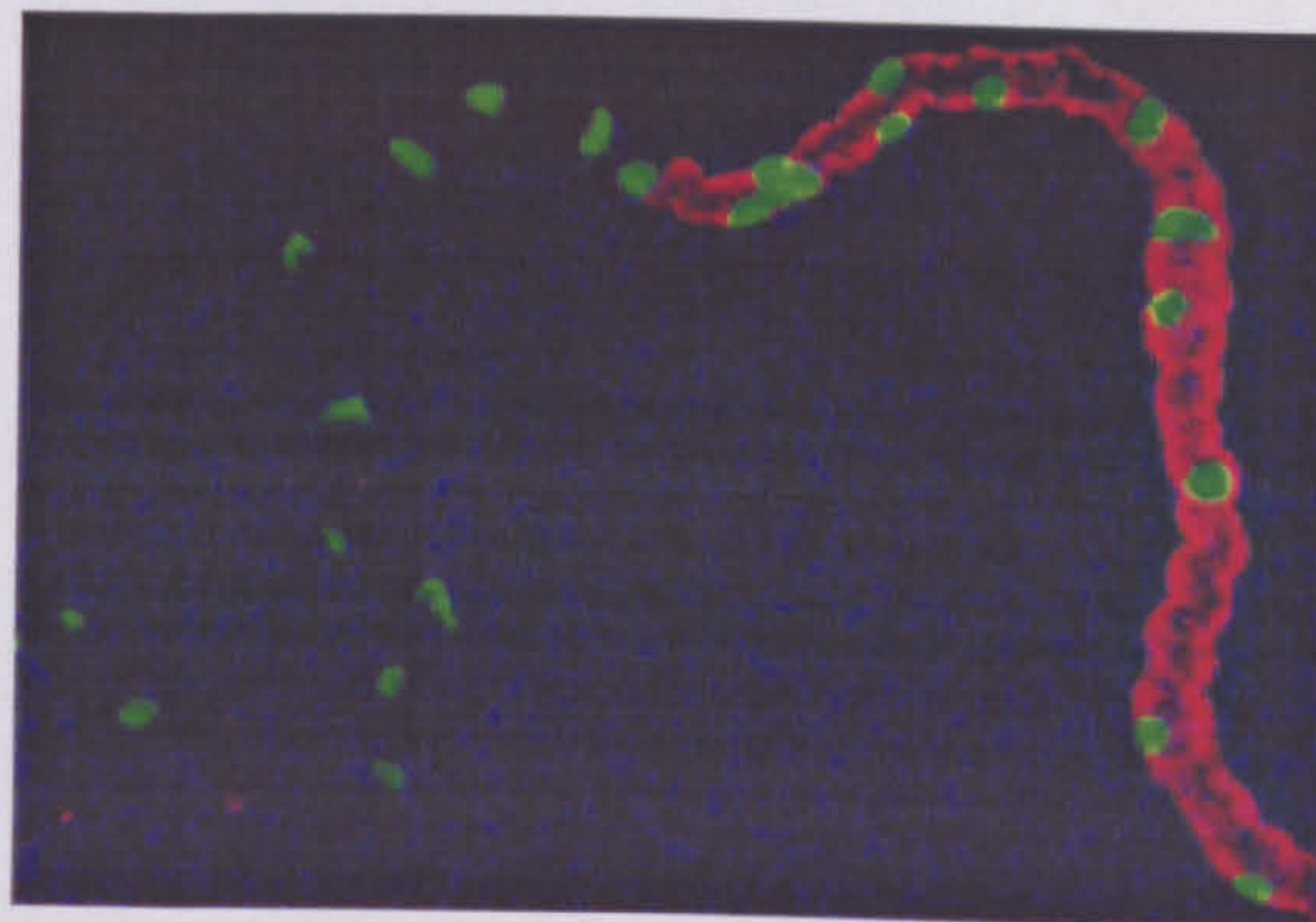
d)



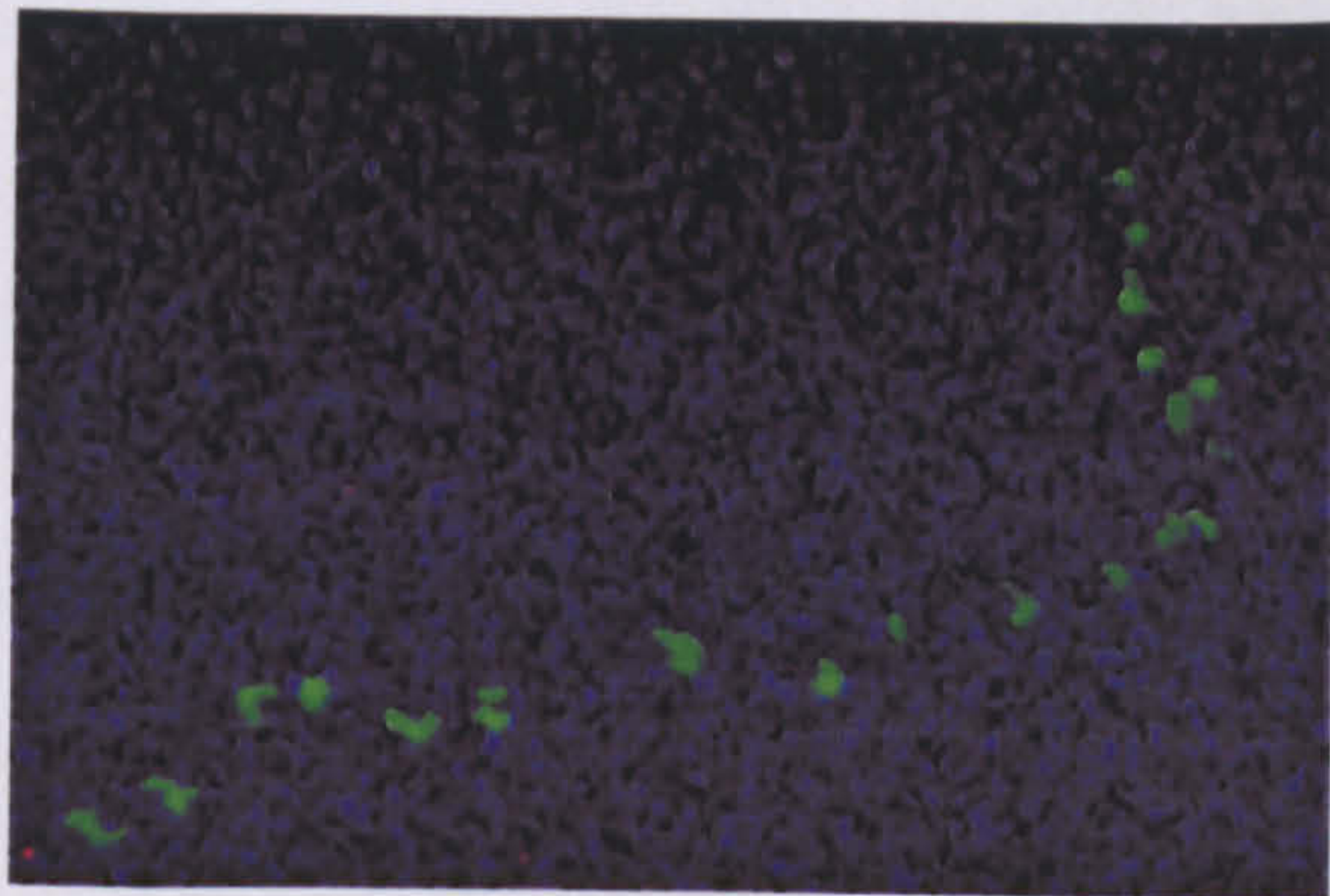
e)



f)



g)



h)

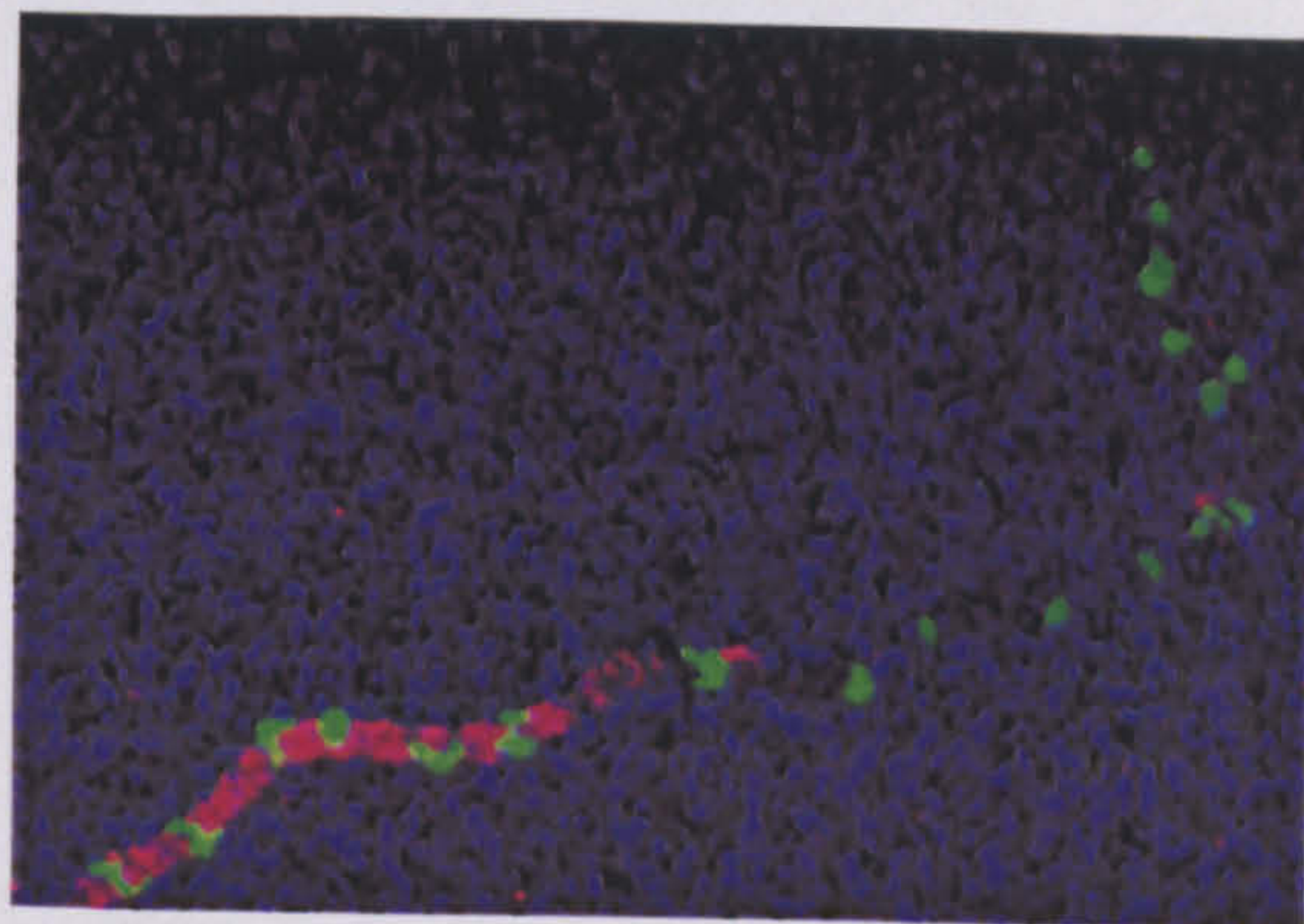
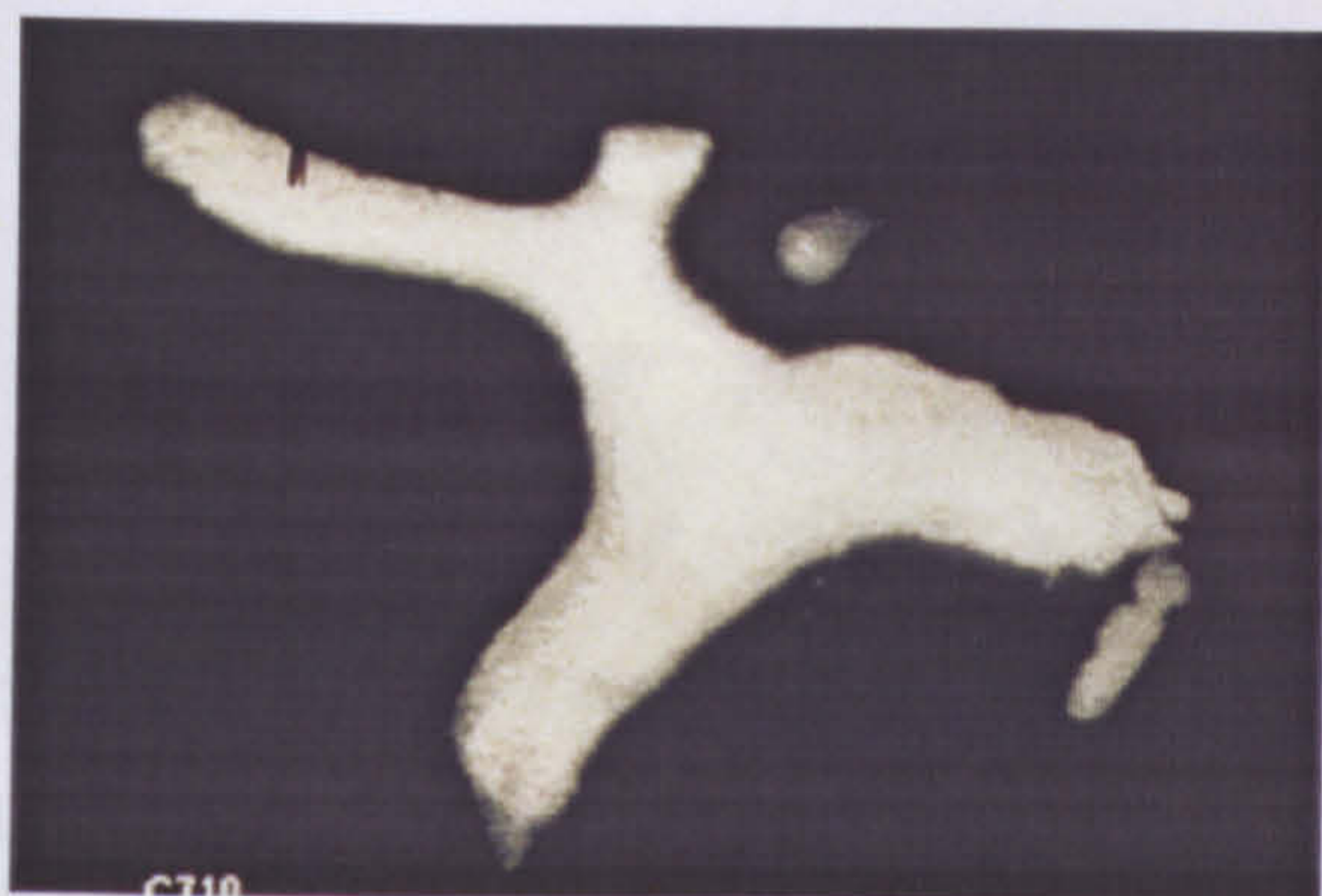


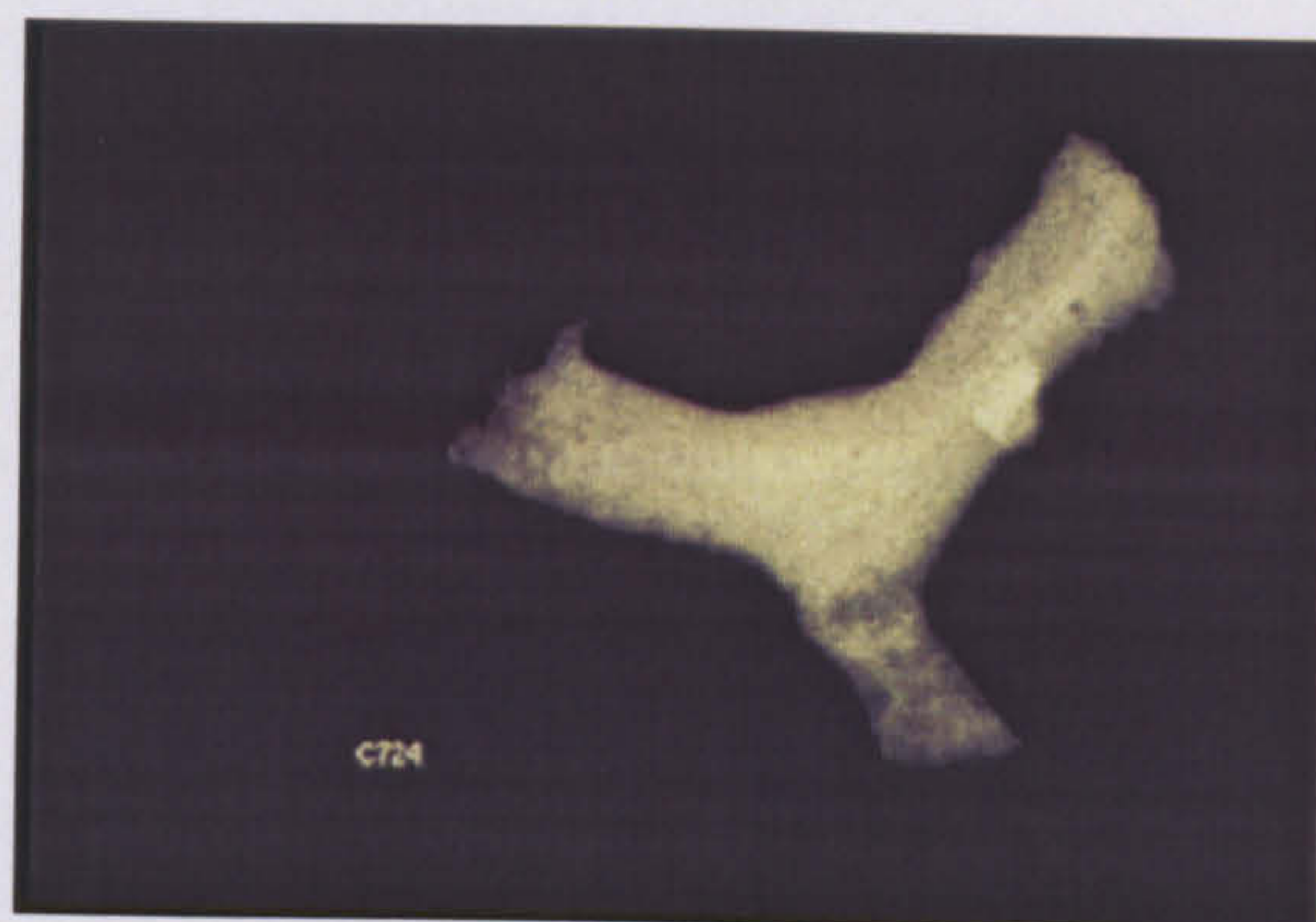
Figure 3.24. Variable morphology of stellate-shaped cells

- a) A single stellate cell with four intercalations among the principal cells (not clearly seen) in line C710 in adult.
 - b) One single stellate cell with three intercalations among the principal cells from line C724 in adult.
 - c) Several stellate cells with their starry shapes in line C724 in adult.
 - d) Four stellate cells in the shape of flower from line C724 in adult.
 - e) The type II cells (marked by line C724) in third instar larvae are not stellate-shaped.
 - f) A single type II cell from main segment of anterior tubule in a third instar larva from line C724.
 - g) A single type II cell from main segment of anterior tubule in a third instar larva in line c724.
 - h) A single type II cell from main segment of posterior tubule in third instar larva from line C710.
-

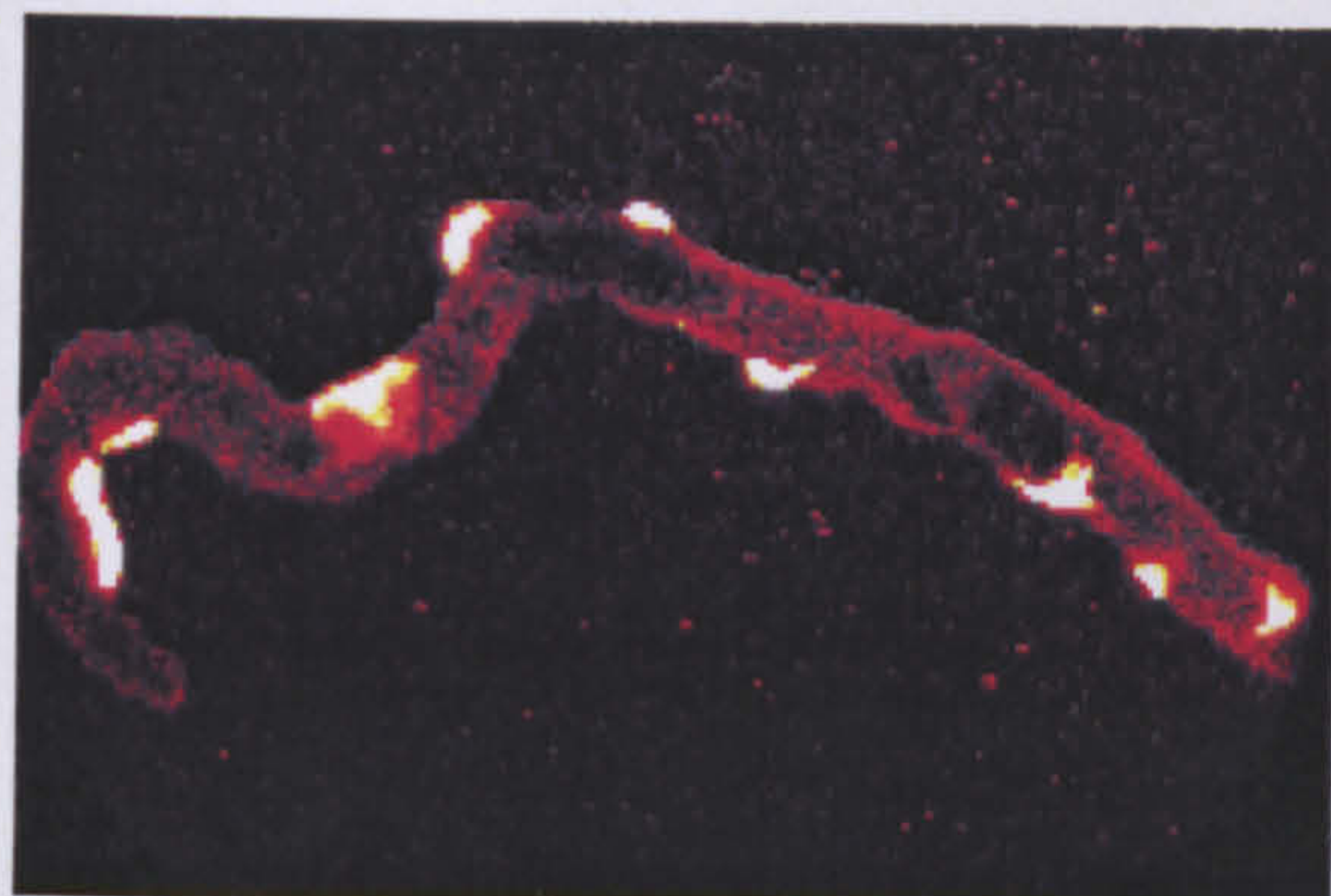
a)



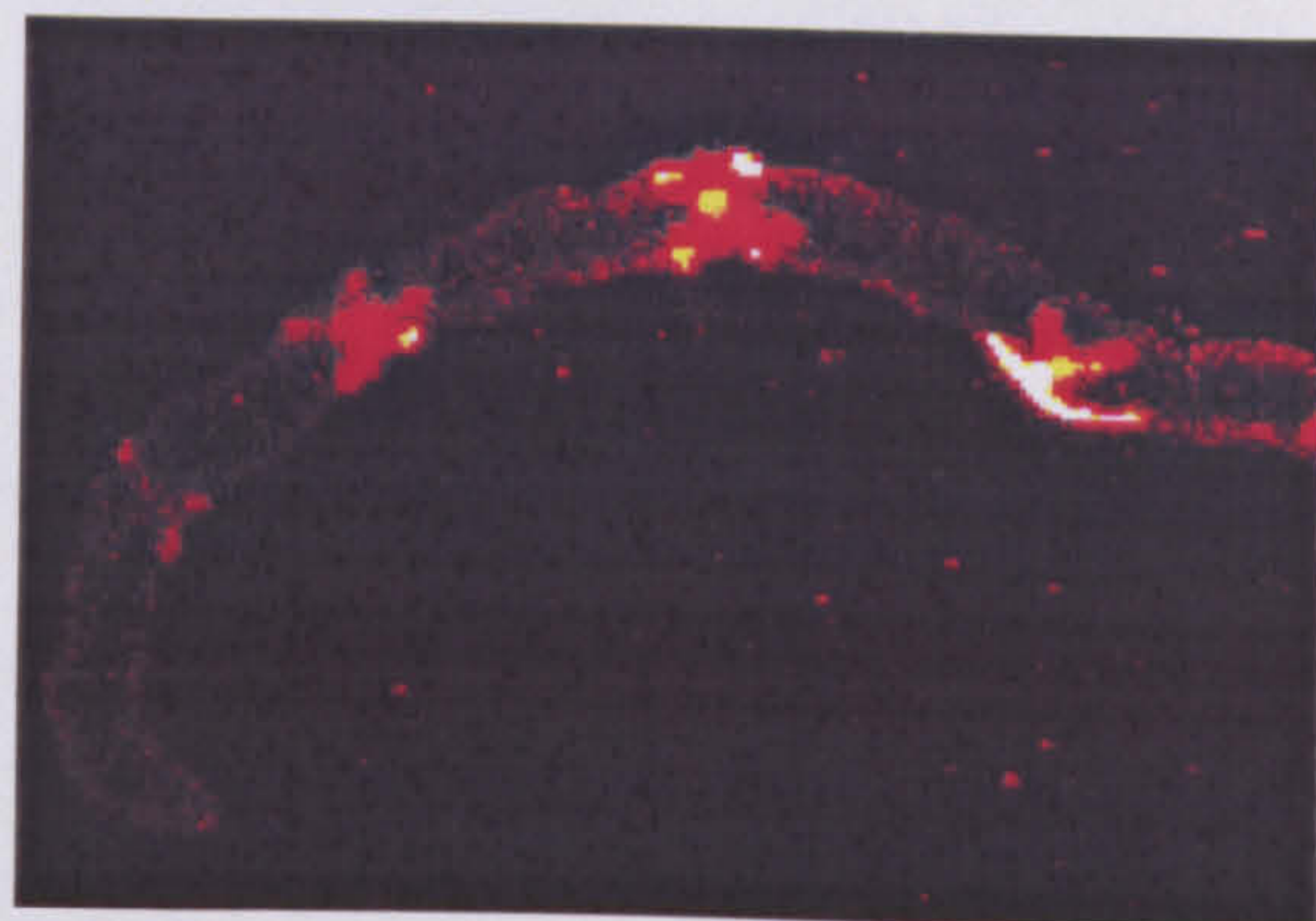
b)



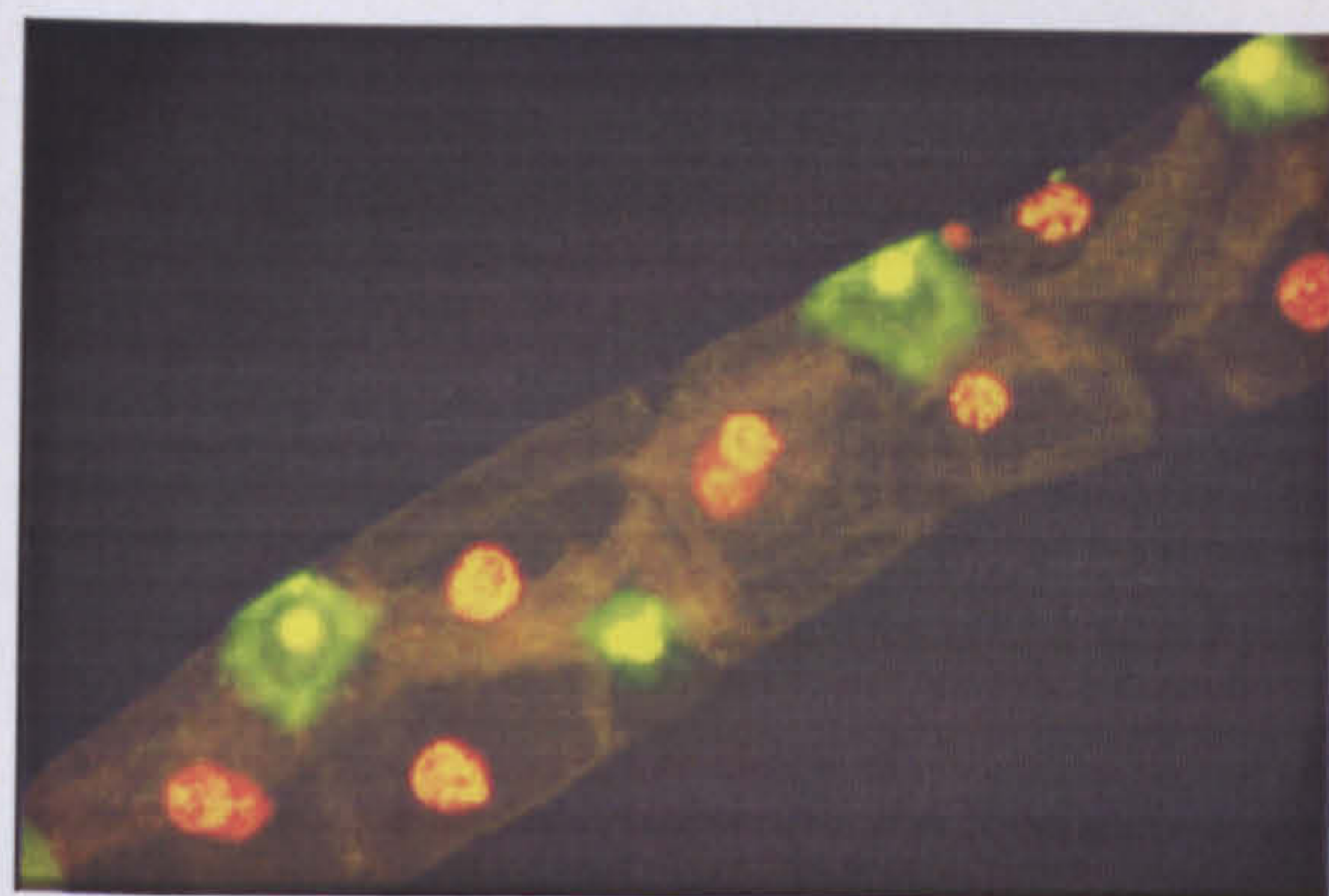
c)



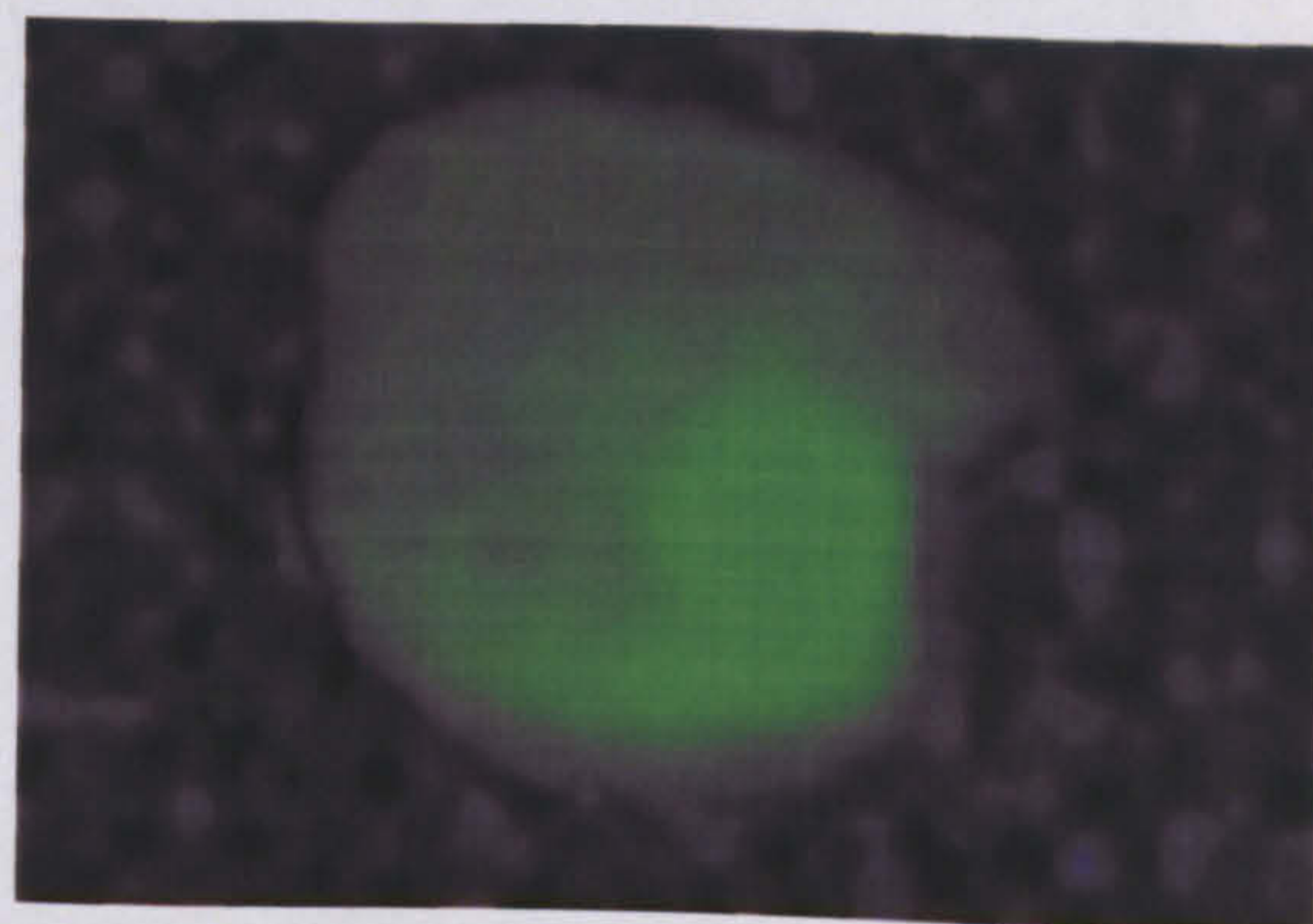
d)



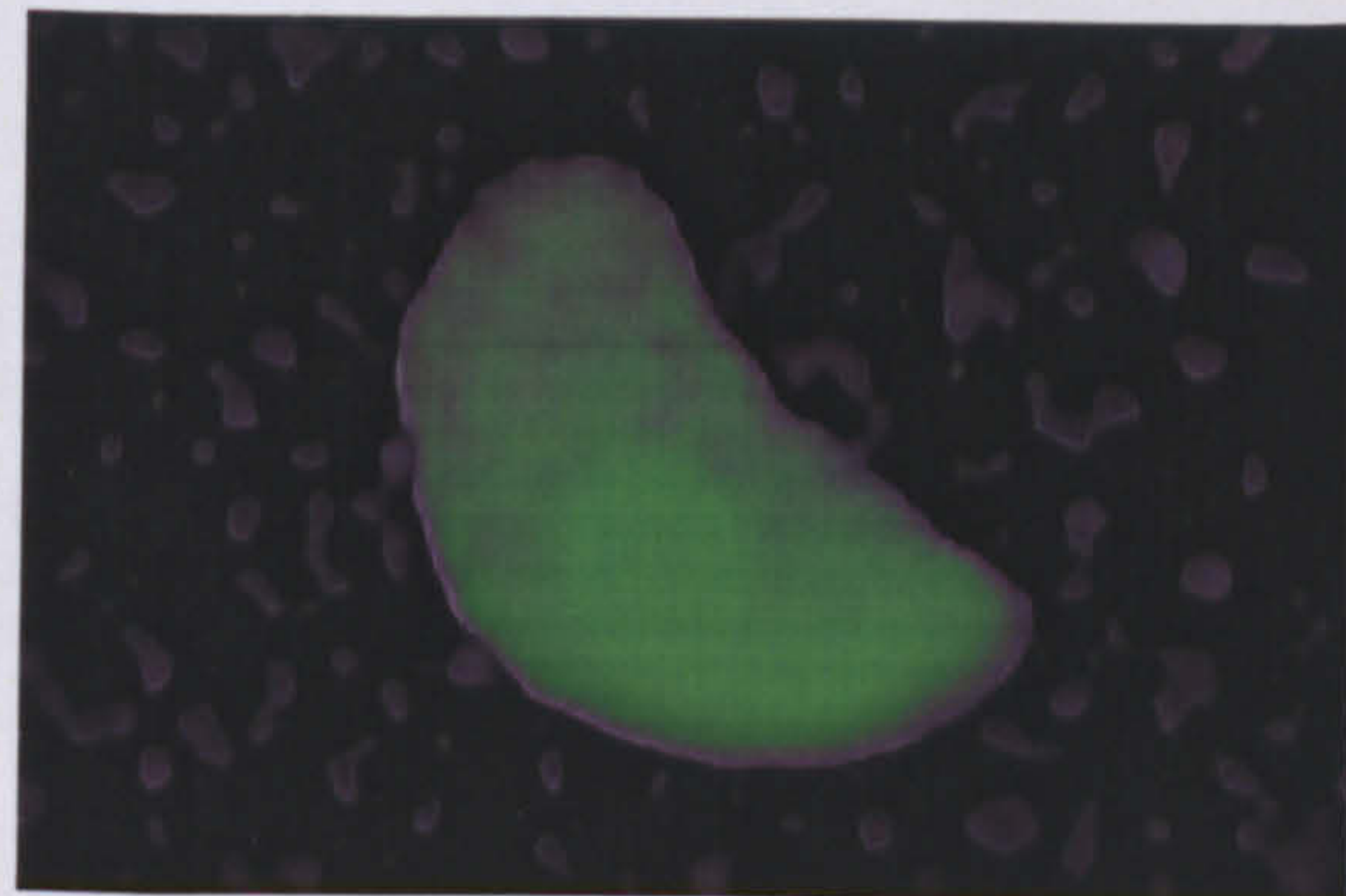
e)



f)



g)



h)

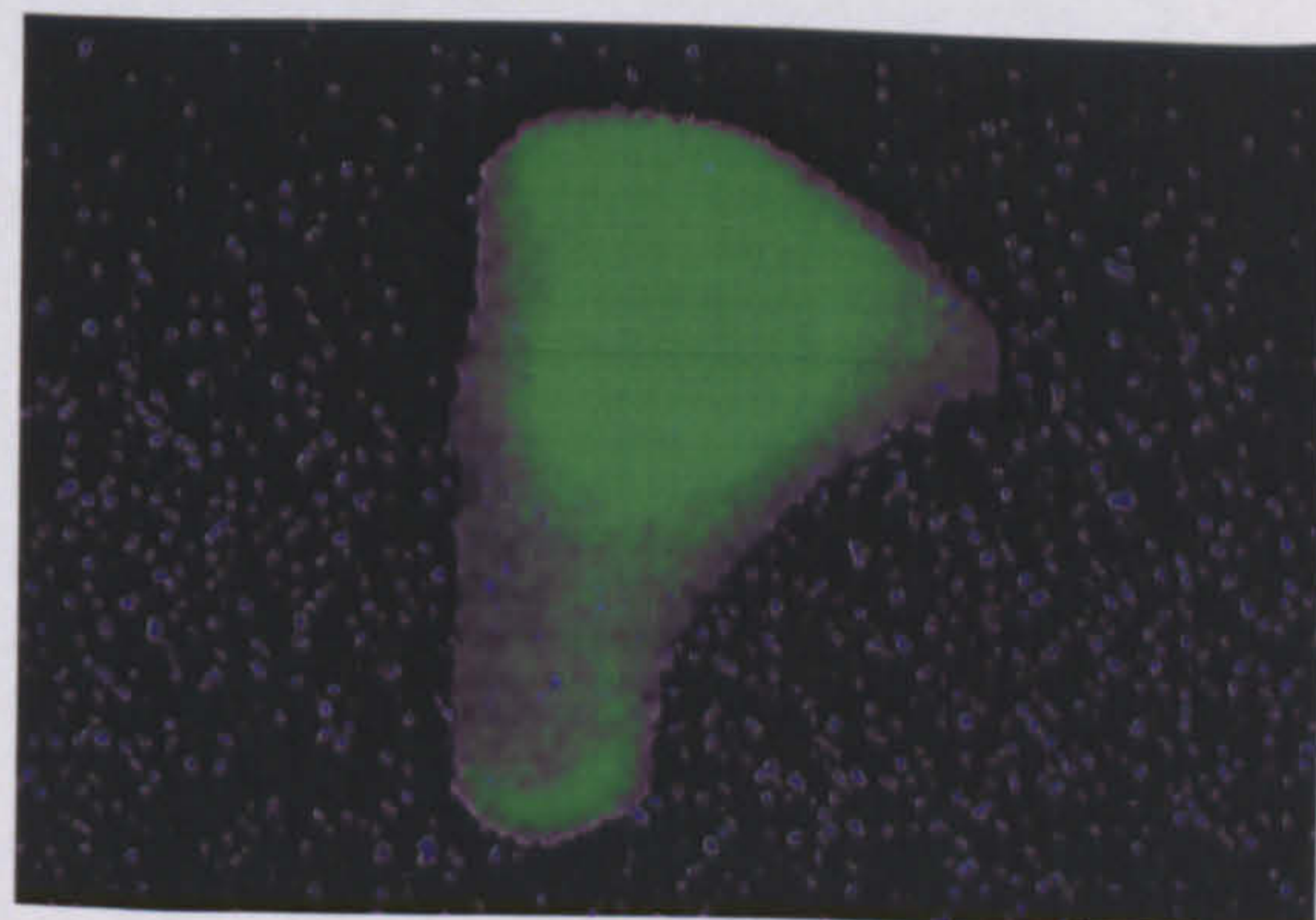
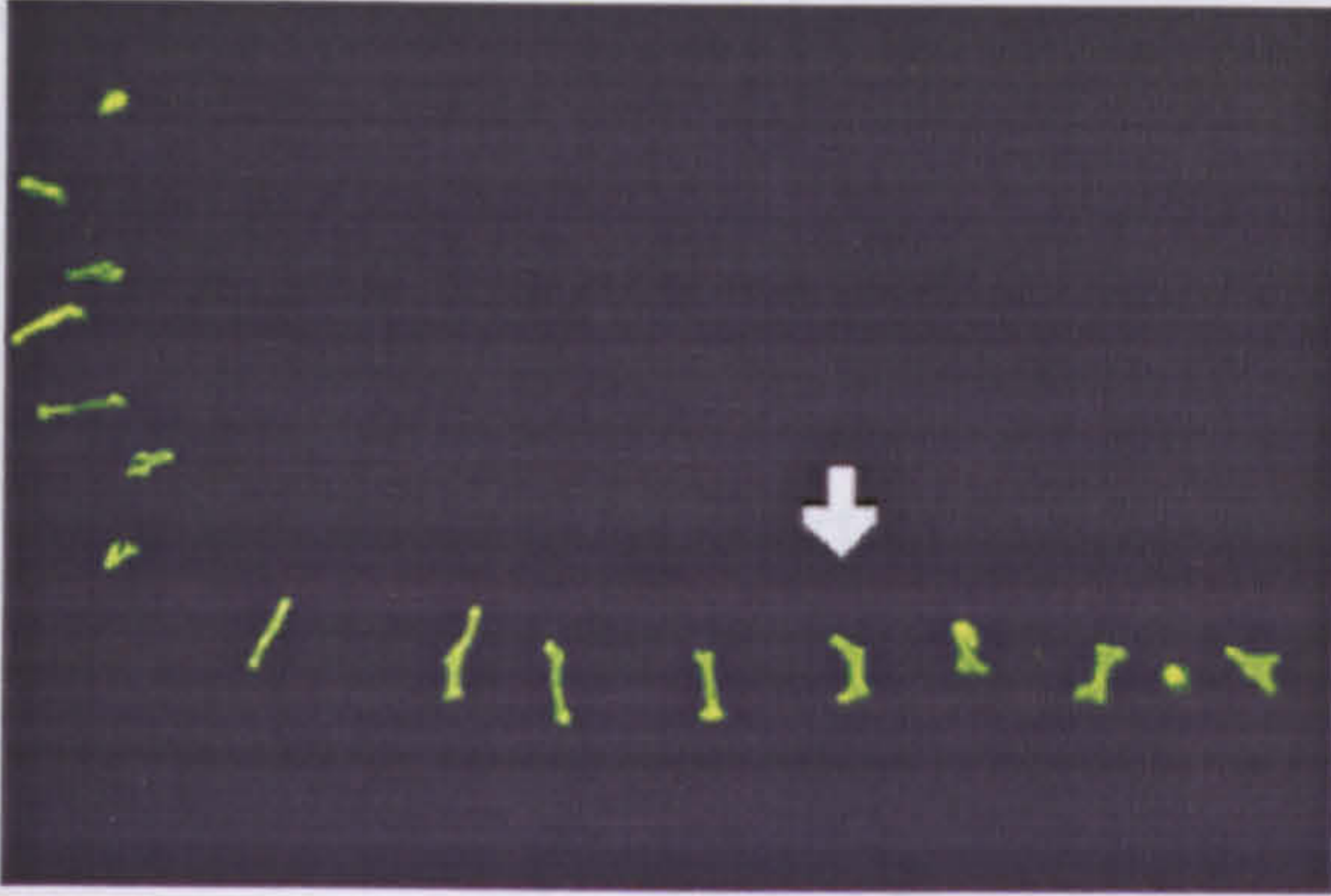


Figure 3.25. Variable morphology of bar-shaped cells

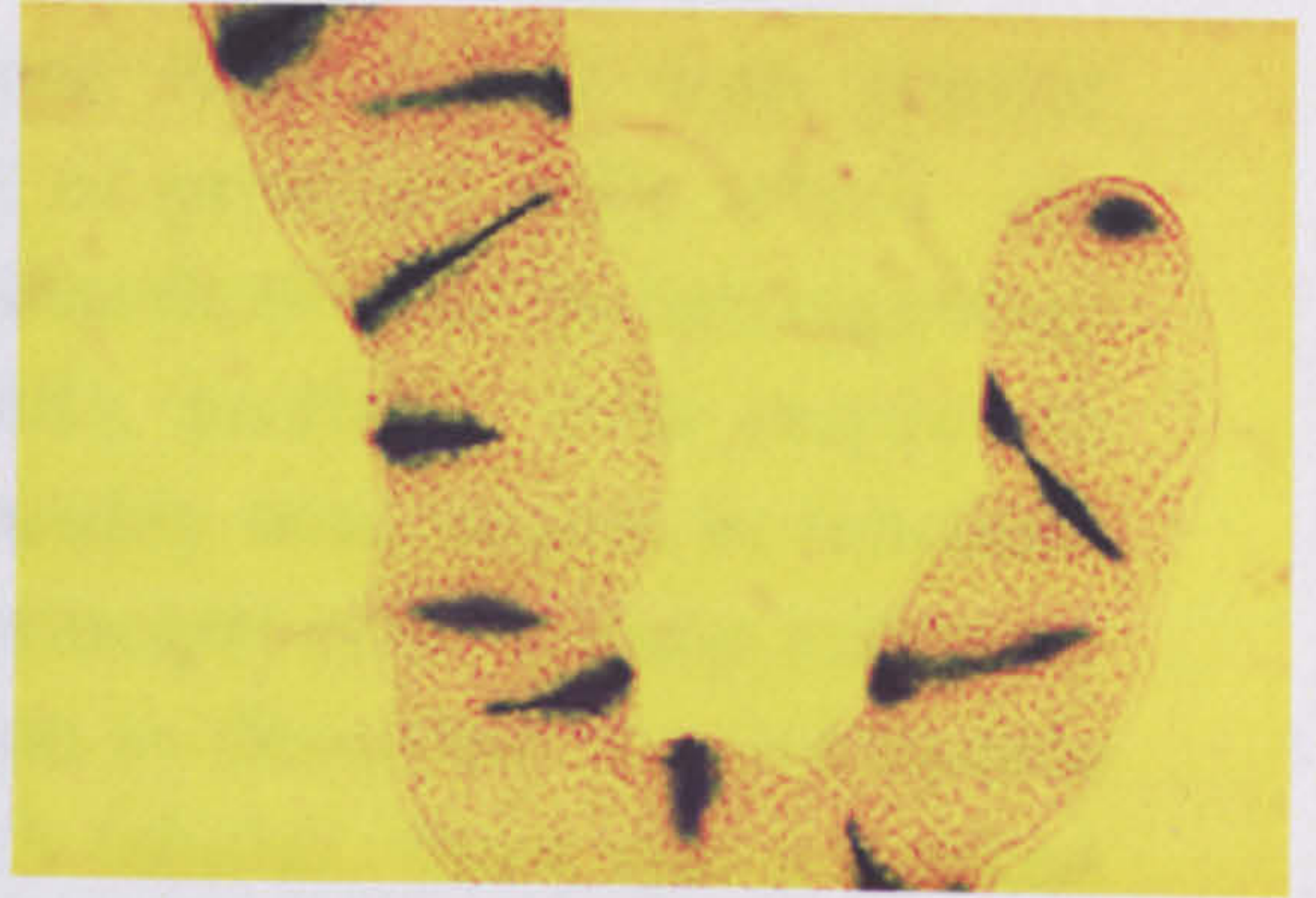
- a) An overall view of all bar-shaped cells of adult anterior Malpighian tubule by antibody staining against β -gal. The boundary where the stellate-shaped cells starts is arrowed. The regularity of their distribution suggests that they could have been generated by lateral inhibition, together with the stellate-shaped cells (Hoch, *et al.*, 1994).
 - b) Most of the bar-shaped-cells of initial and transitional segment by X-gal staining in adult from line C724. This line frequently labels a single cell at the tip of the tubule.
 - c) Three bar-shaped cells at higher magnification by GAL4-directed GFP expression from line C649.
 - d) Differentiating bar-shaped cells at the pupal stage are closer to adult . Compare (a-c) with (e-h).
 - e) Three bar-shaped cells of initial and transitional segment at third instar larval stage are similar to those of the adult but not completely bar-shaped yet.
 - f) A single bar-shaped cell of larva at higher magnification.
 - g) A couple of bar-shaped cells of anterior tubule in third instar larva are sometimes even close to square-shaped morphology and slightly larger than the main segment stellate-shaped cells.
 - h) One single bar-shaped cell of larva (3rd instar) at higher magnification.
-

3.7.2. The effect of temperature and time on *lacZ* expression.

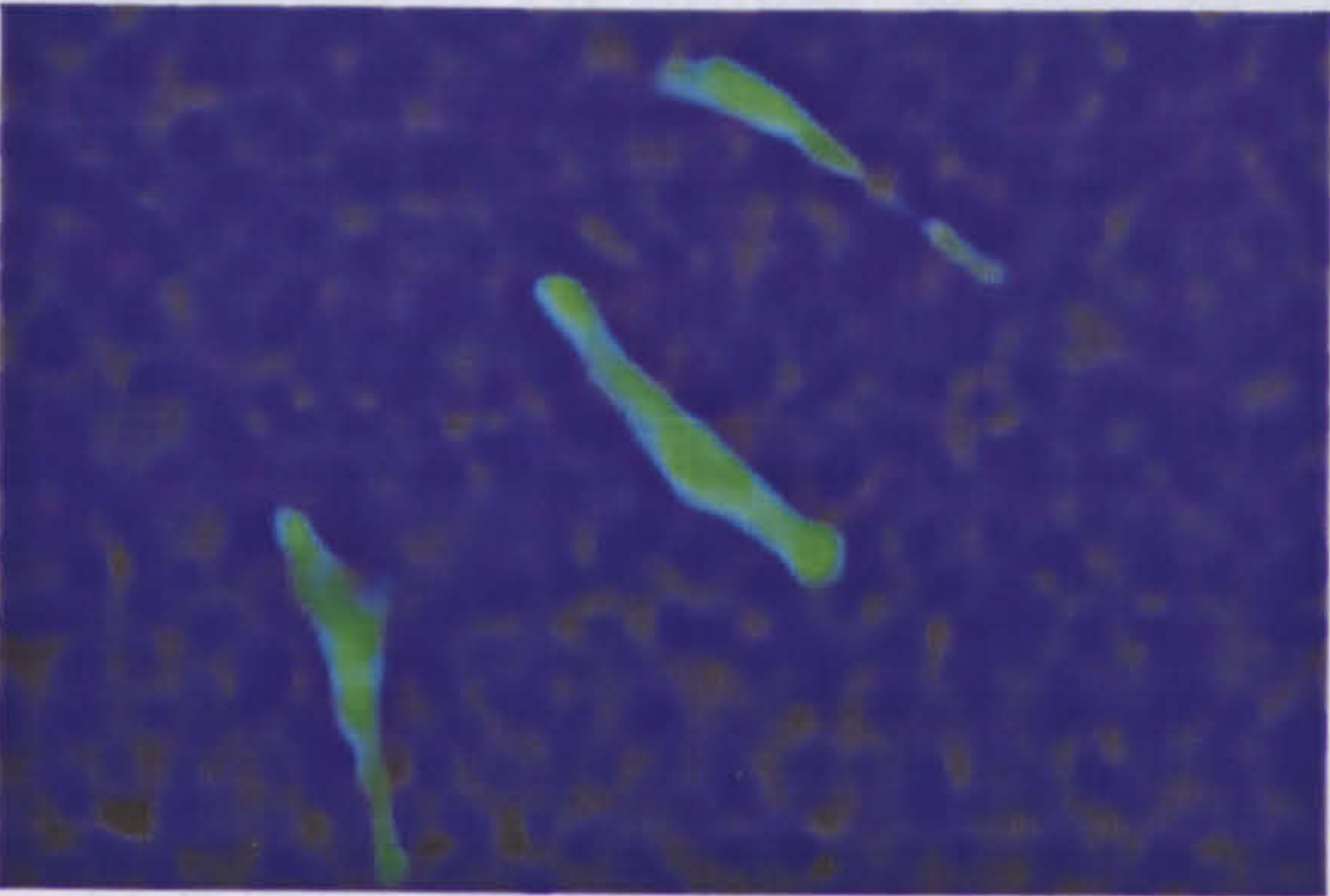
a)



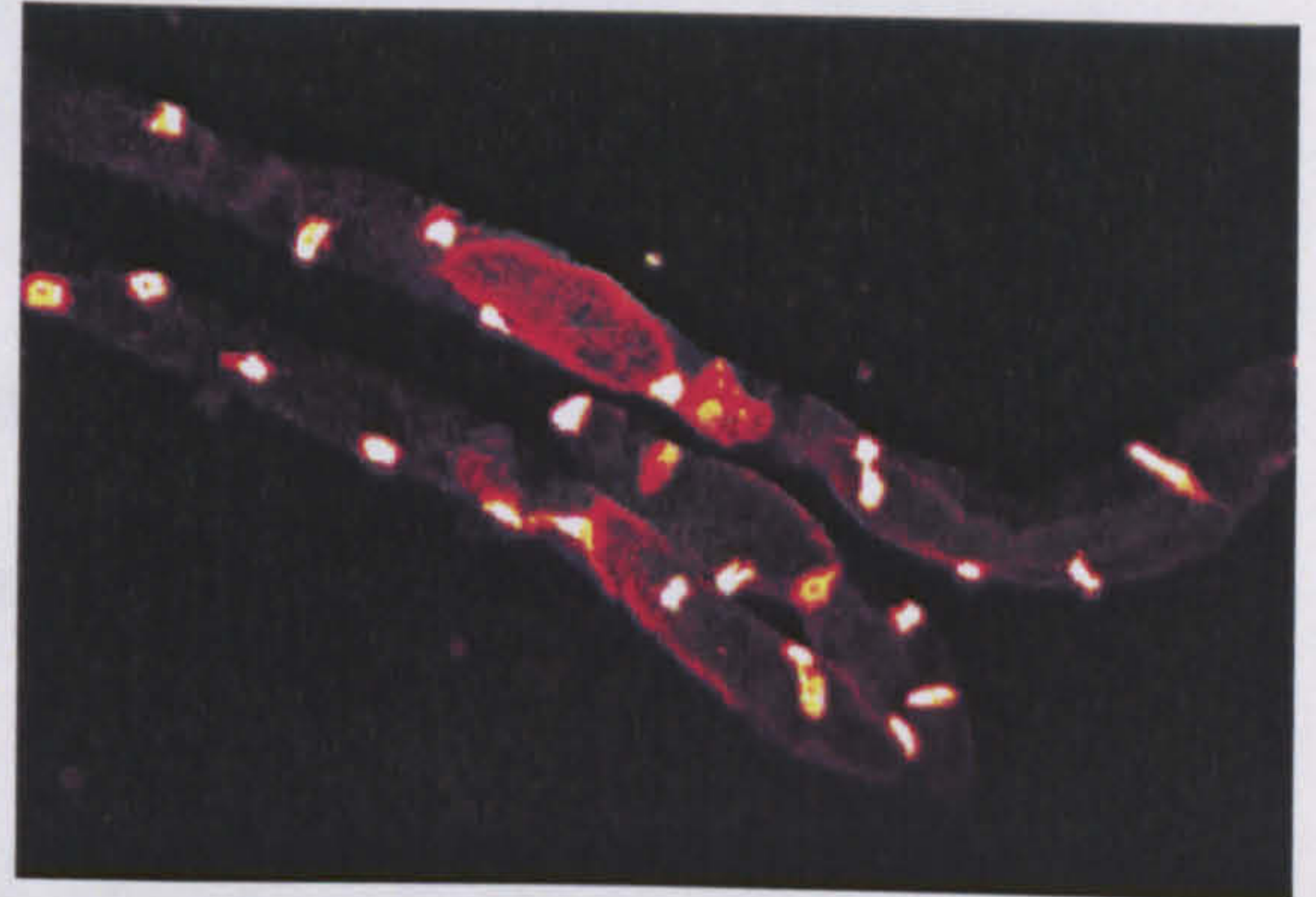
b)



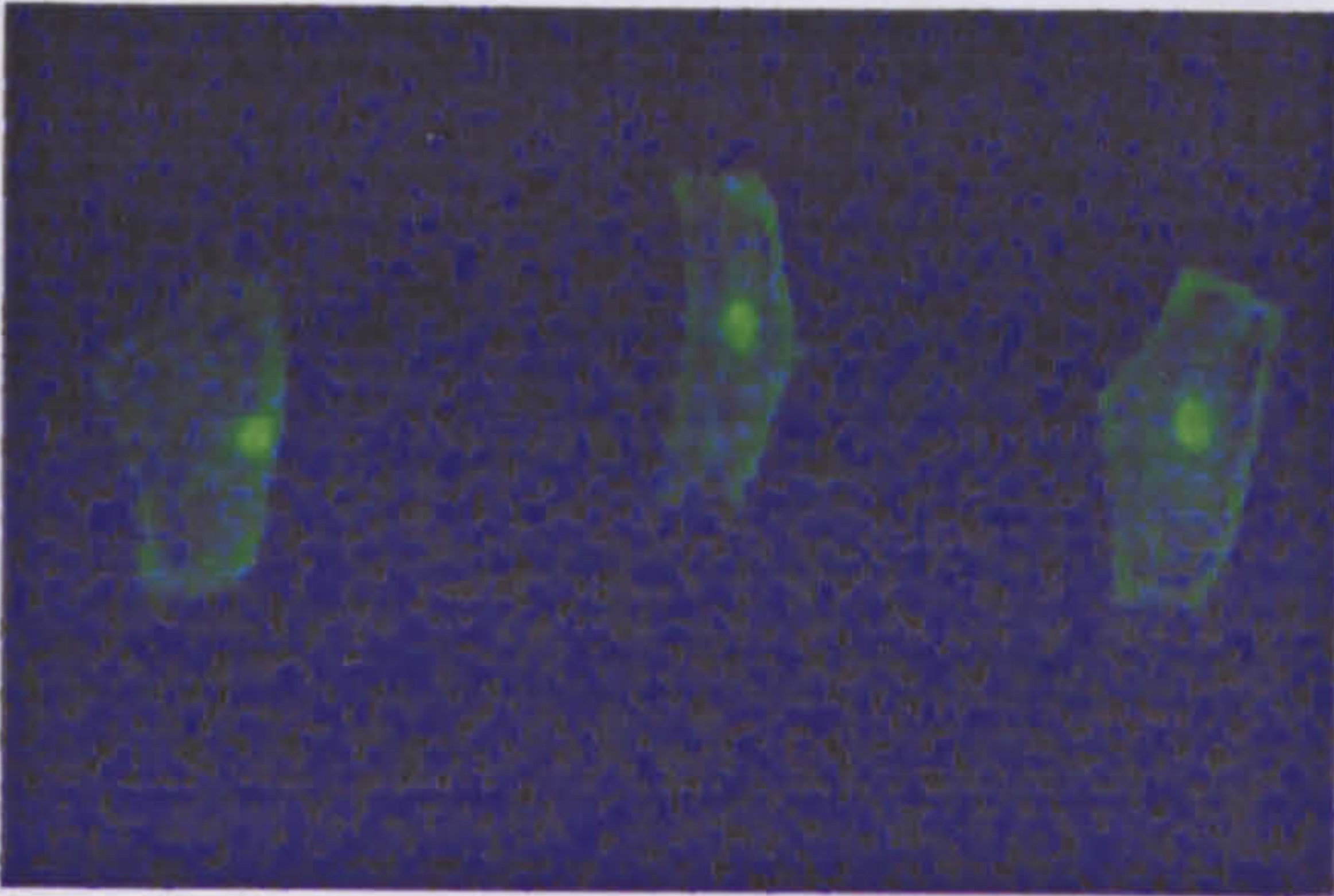
c)



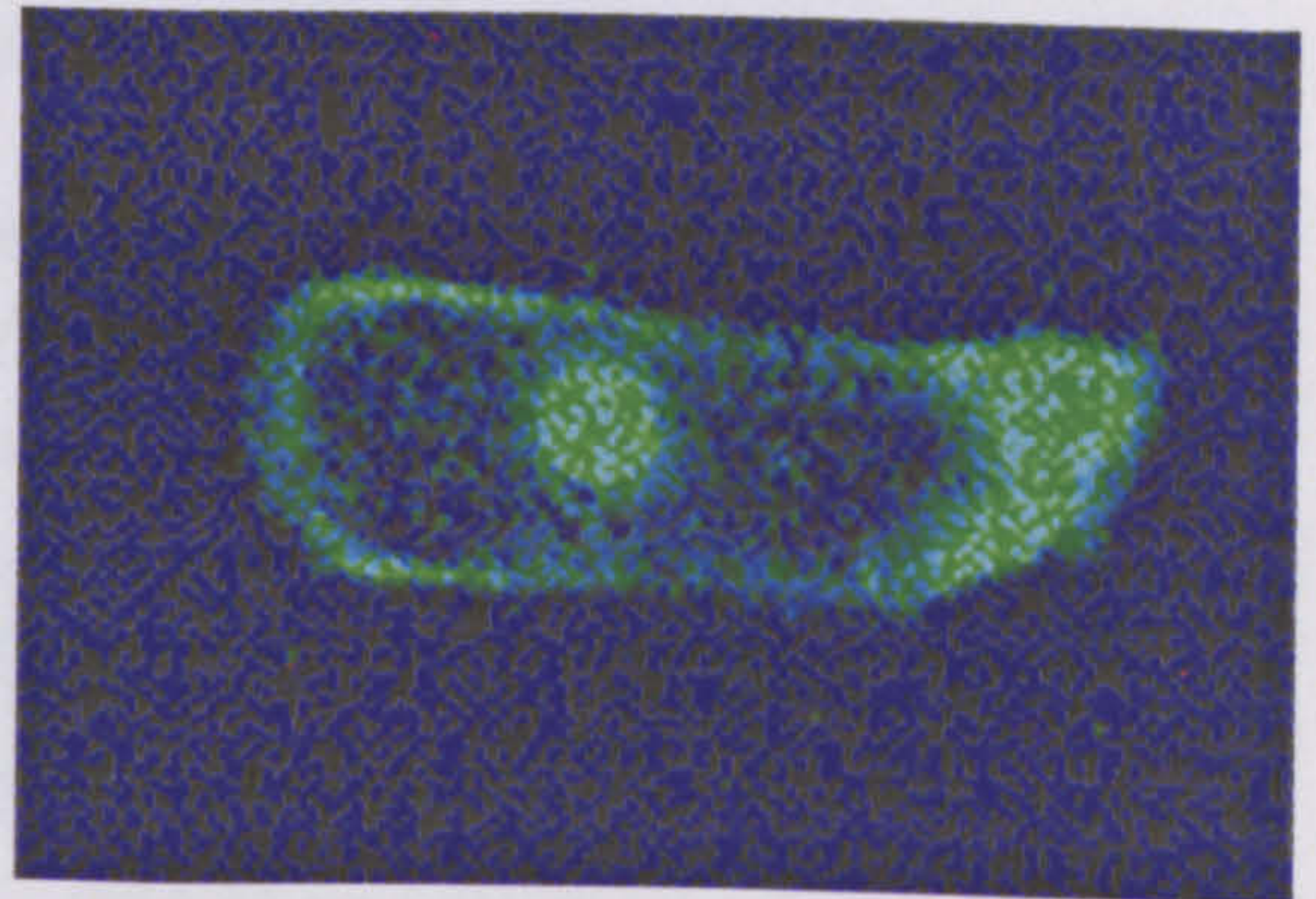
d)



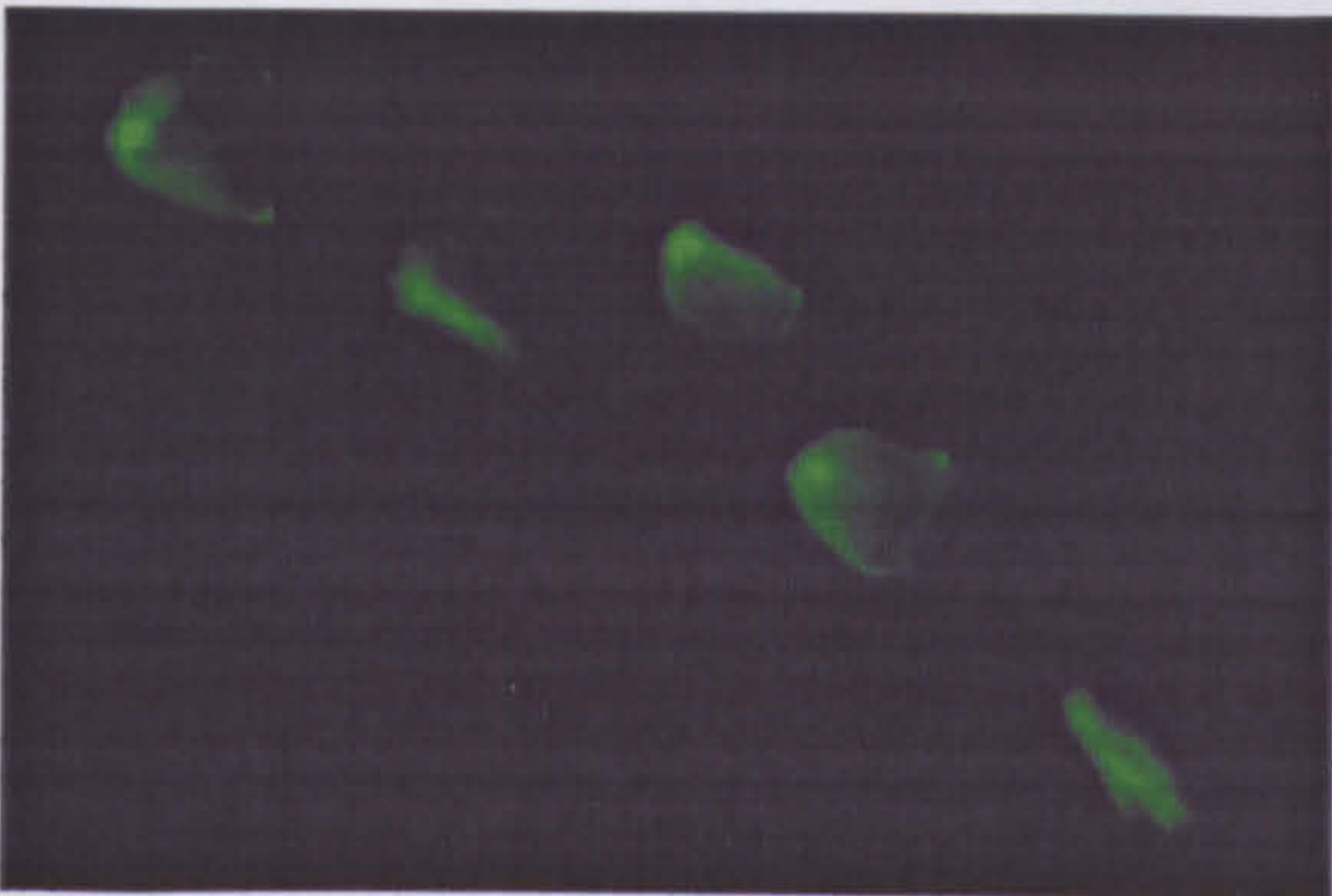
e)



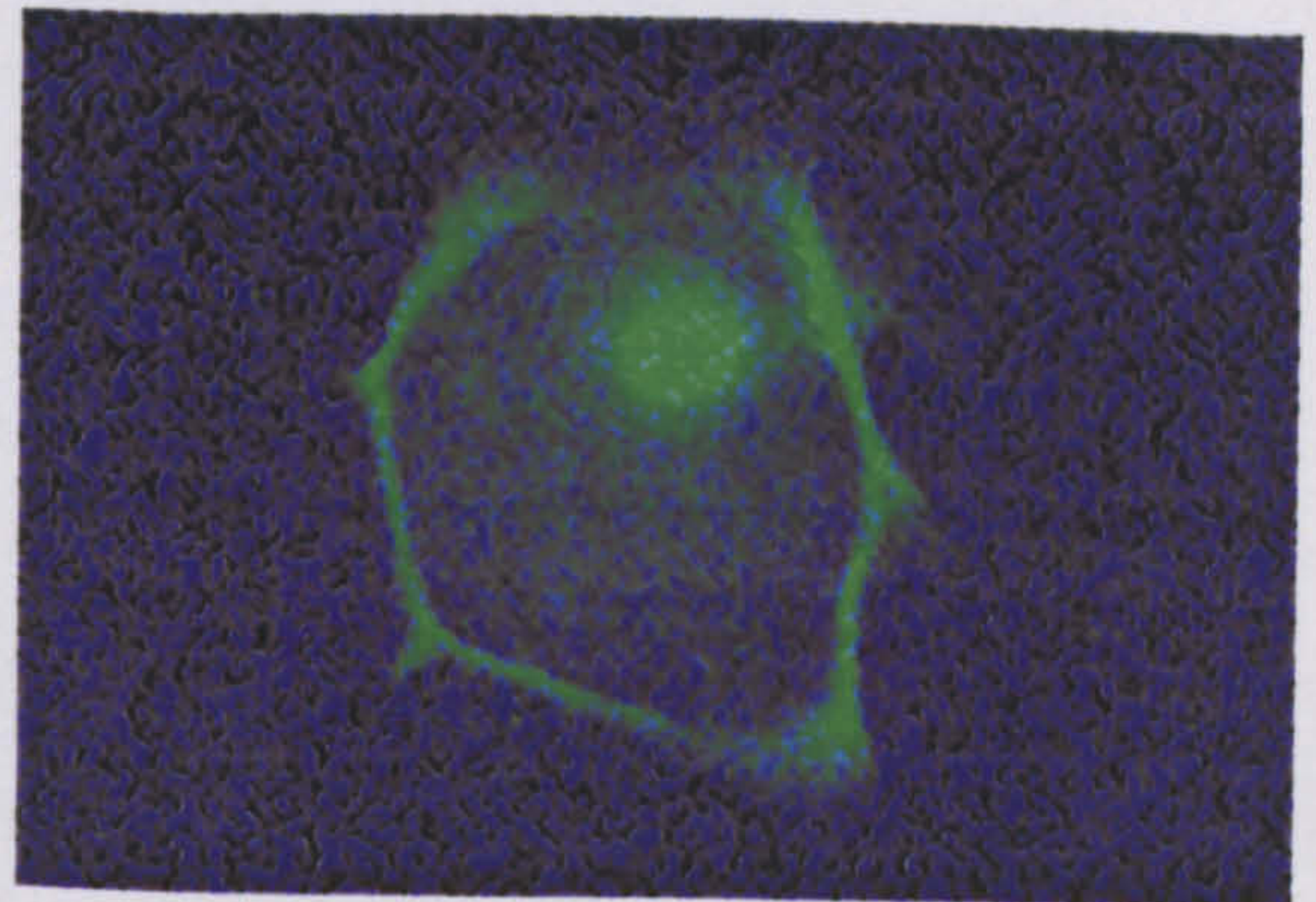
f)



g)



h)



3.7.2. The effect of temperature and sex on variegated expression.

Several independent enhancer trap lines were found with similar expression patterns in a variable subset of principal cells which are otherwise indistinguishable. This implies that there must be heterogeneity among otherwise identical principal cells, probably with respect to transcriptional properties, and also presumably with respect to function. The cause of the mosaicism among the principal cells in these enhancer trap lines is not position effect variegation as described for *Drosophila white* locus (Van Breugel, 1973). In such tubules, there was a marked temperature sensitivity. In case of an analogous phenomenon, it had been reported for tubule cells of a class of *white* mutant that showed position effect variegation.

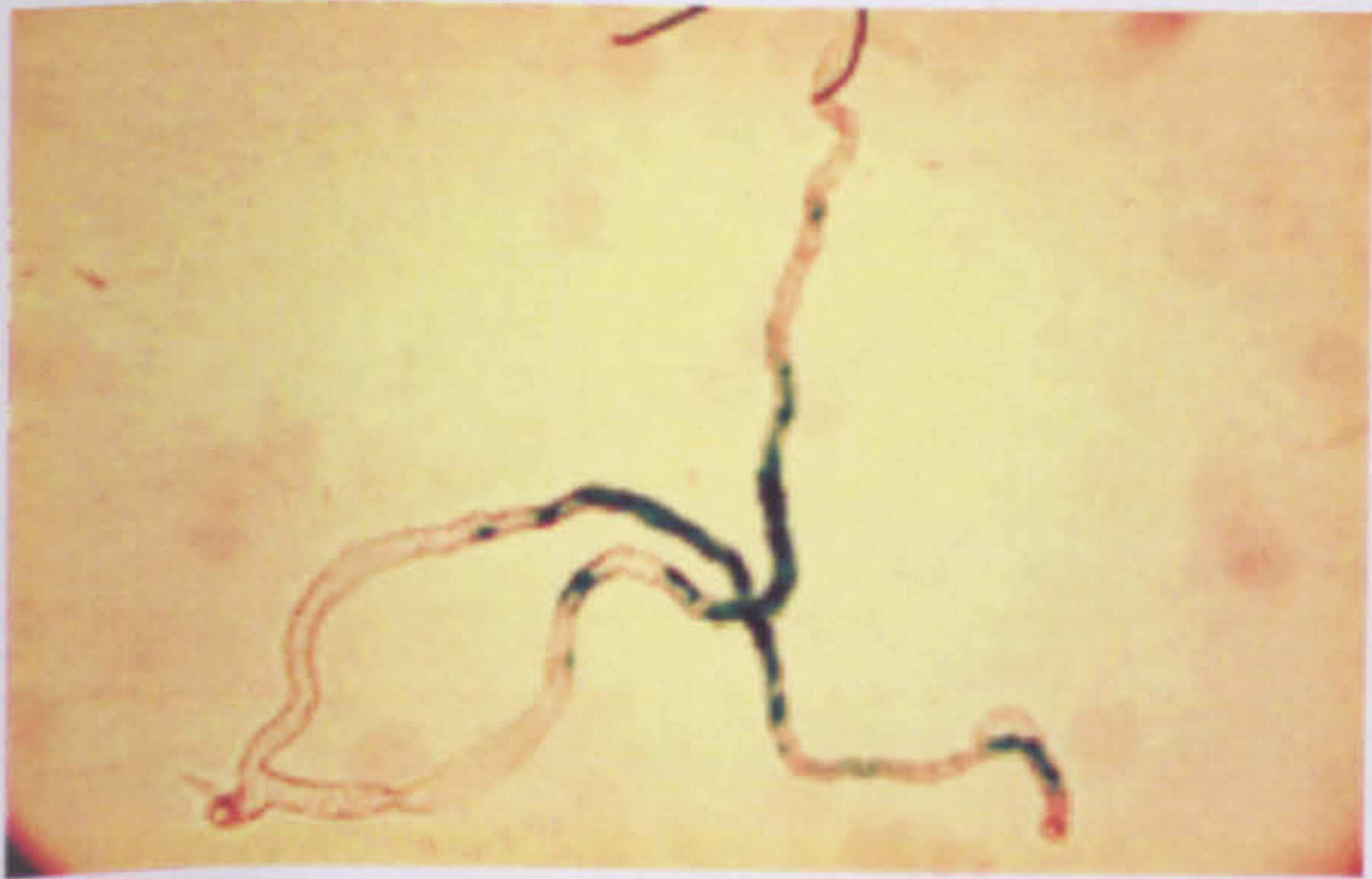
The findings observed in this study can be concluded as follows:

- 1) Lines staining principal cells always seemed to stain a variable subset of such cells.
- 2) It was not thought that position effect variegation accounted for our findings, as the eyes of these flies were not also variegated.

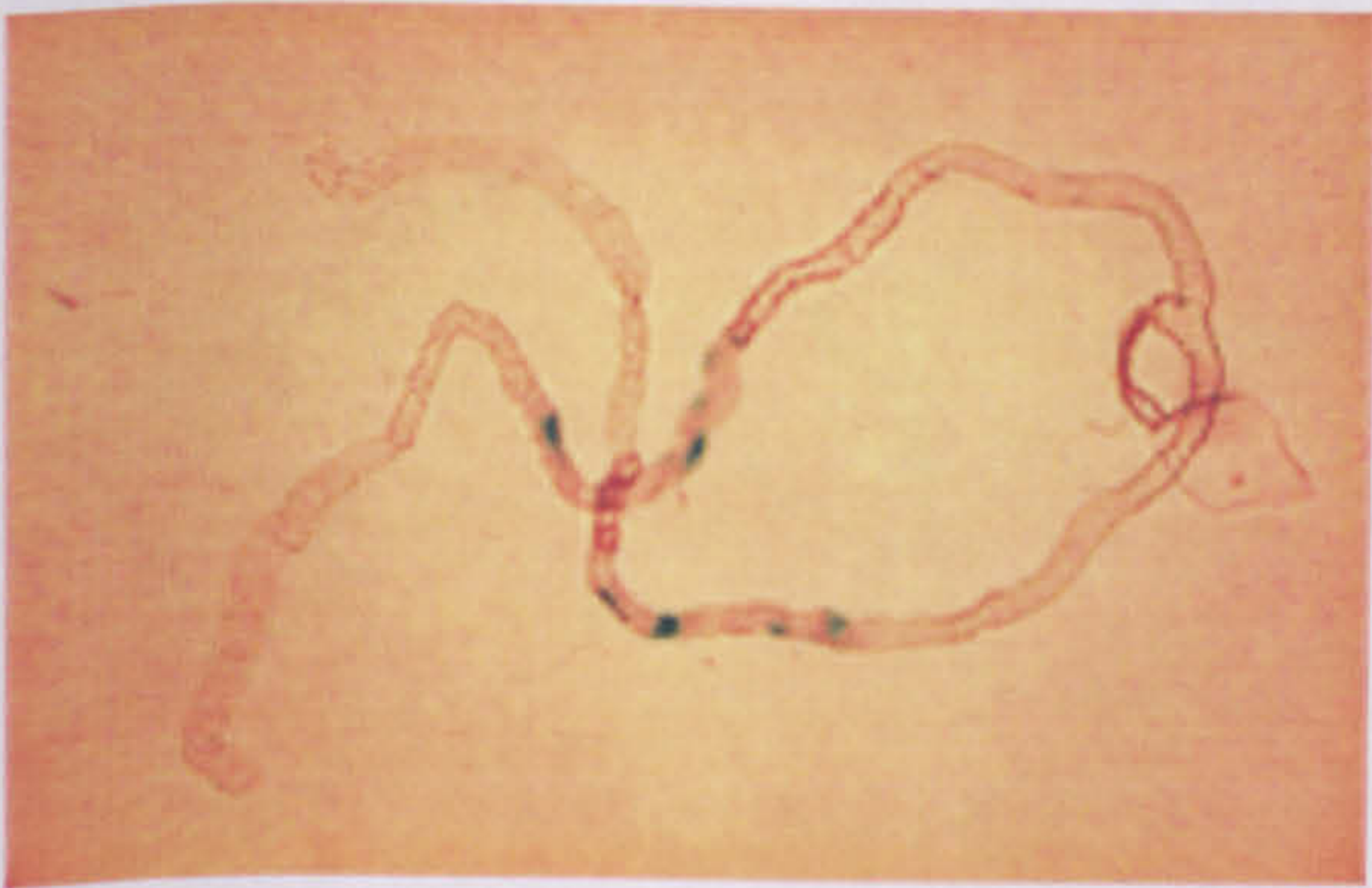
In light of these findings, a study was undertaken to see whether temperature or sex influenced the fraction of principal cells expressing GAL4. Although the variegation described by Van Breugel was exquisitely temperature sensitive, that detected by line C324, C374 and C855 was not, however, a sex-specific effect was observed with a greater number of cells stained in tubules of males than females.

Figure 3.26. The effect of temperature on variegated expression .

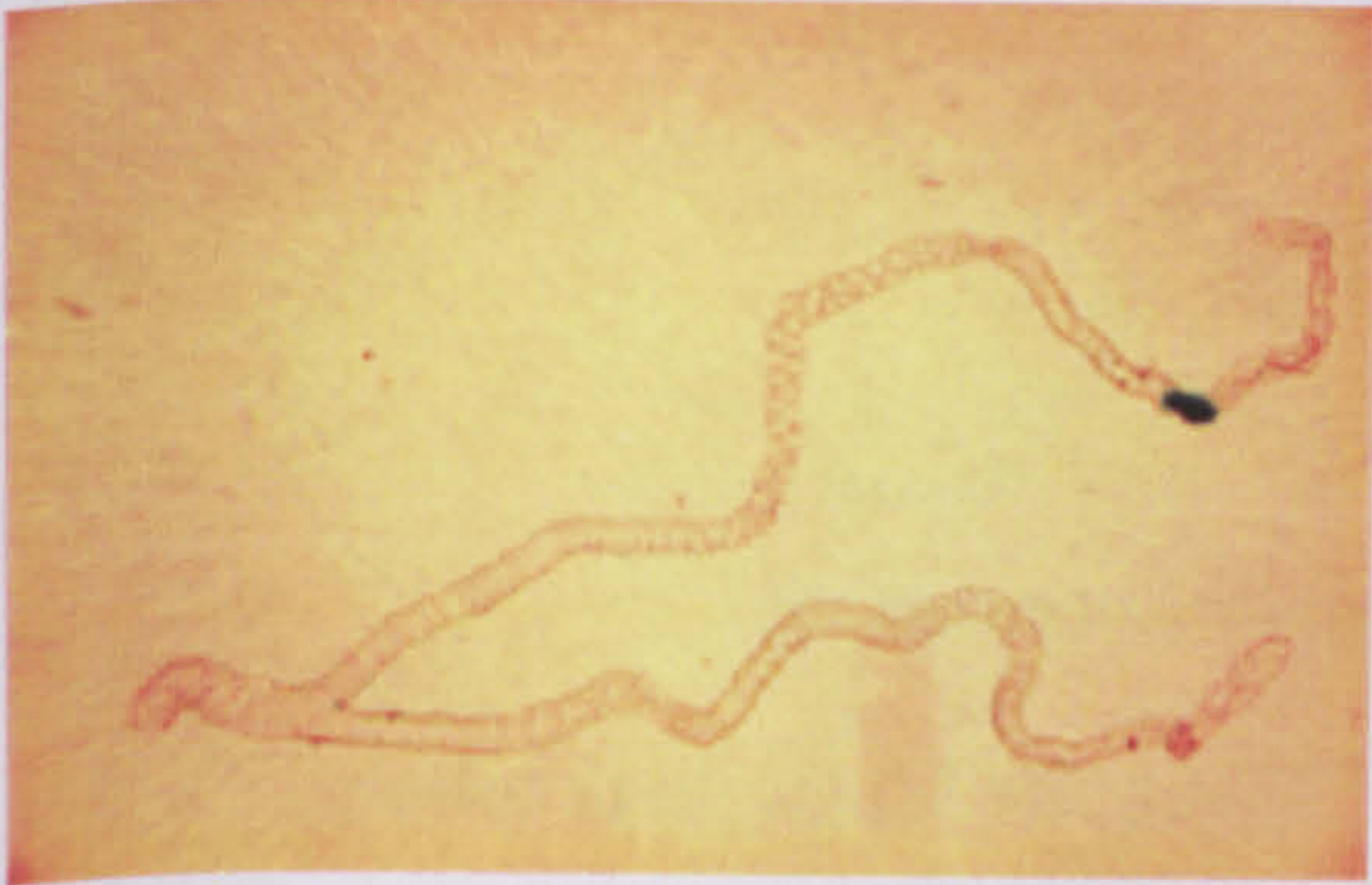
- a) GAL4 expression in line c374 at 18°C.
 - b) GAL4 expression in line c374 at 25°C.
 - c) GAL4 expression in line c855 at 18°C
 - d) GAL4 expression in line c855 at 25°C.
 - e) GAL4 expression in line c324 at 18°C.
 - f) GAL4 expression in line c324 at 25°C.
 - g) GAL4 expression in the secondary cells (Bertram, et al., 1992) of male accessory glands at 18°C in line c324.
 - h) The expression in the secondary cells of male accessory glands at 25°C in line c324.
-



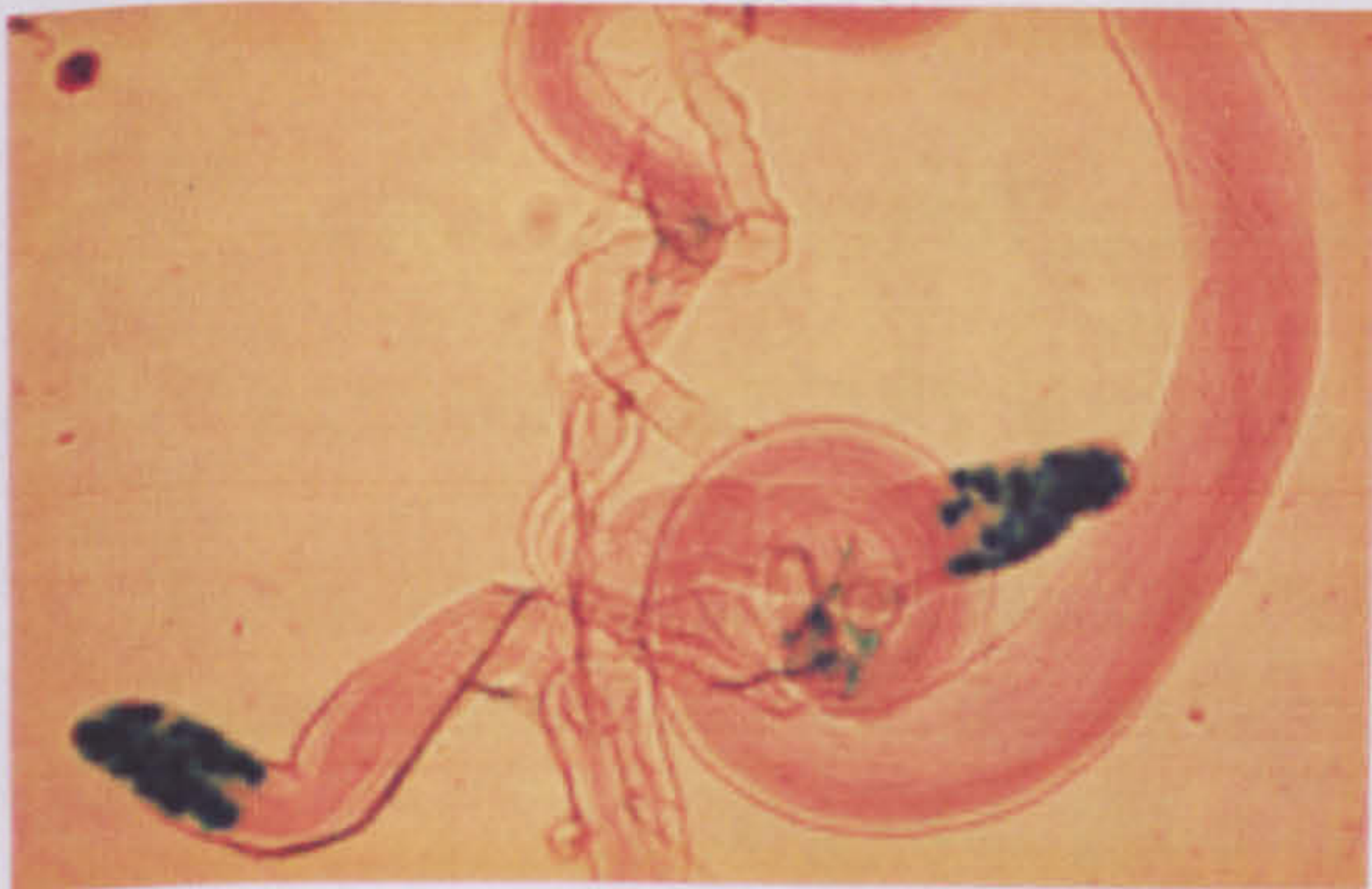
b)



d)



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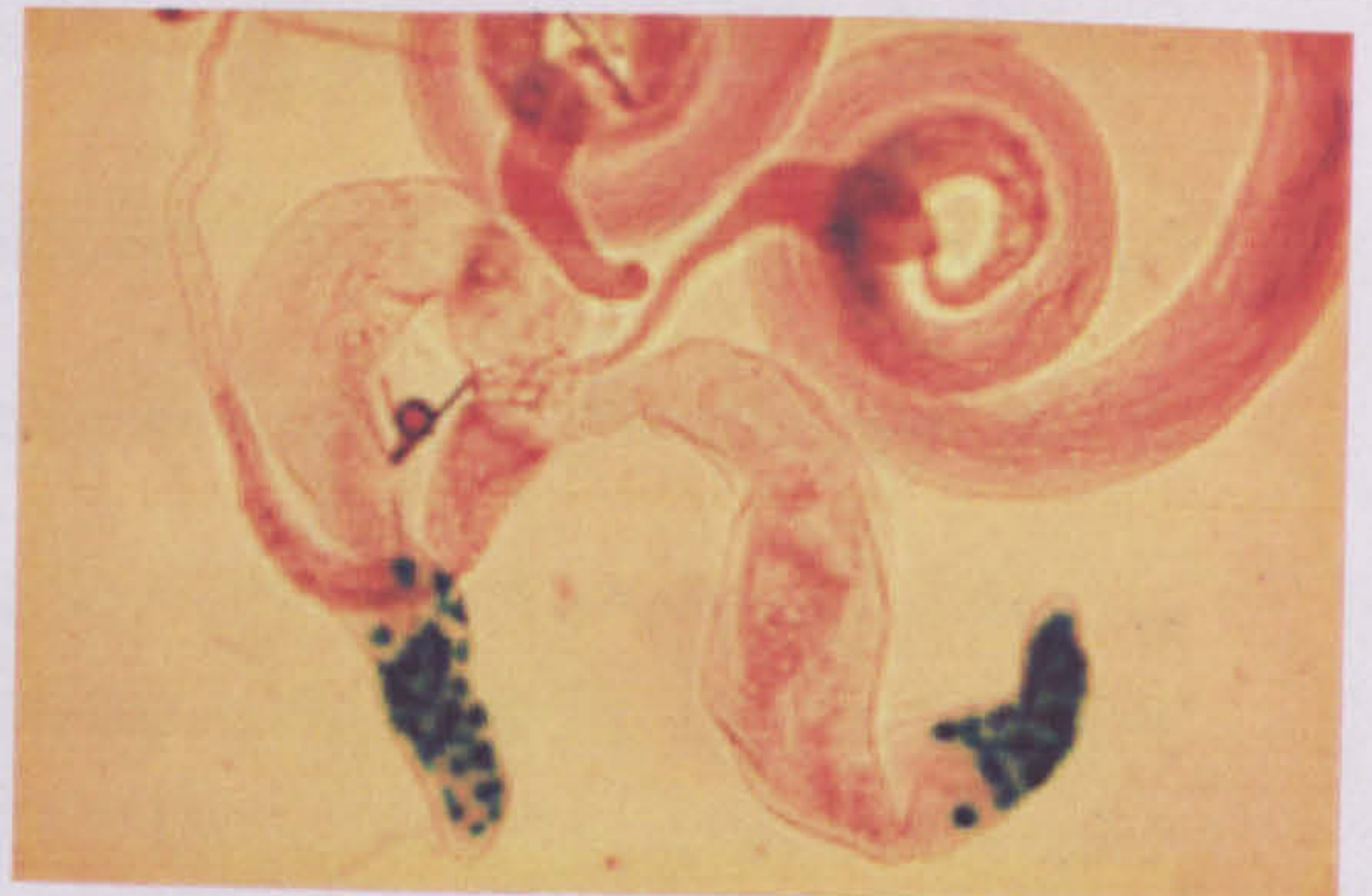
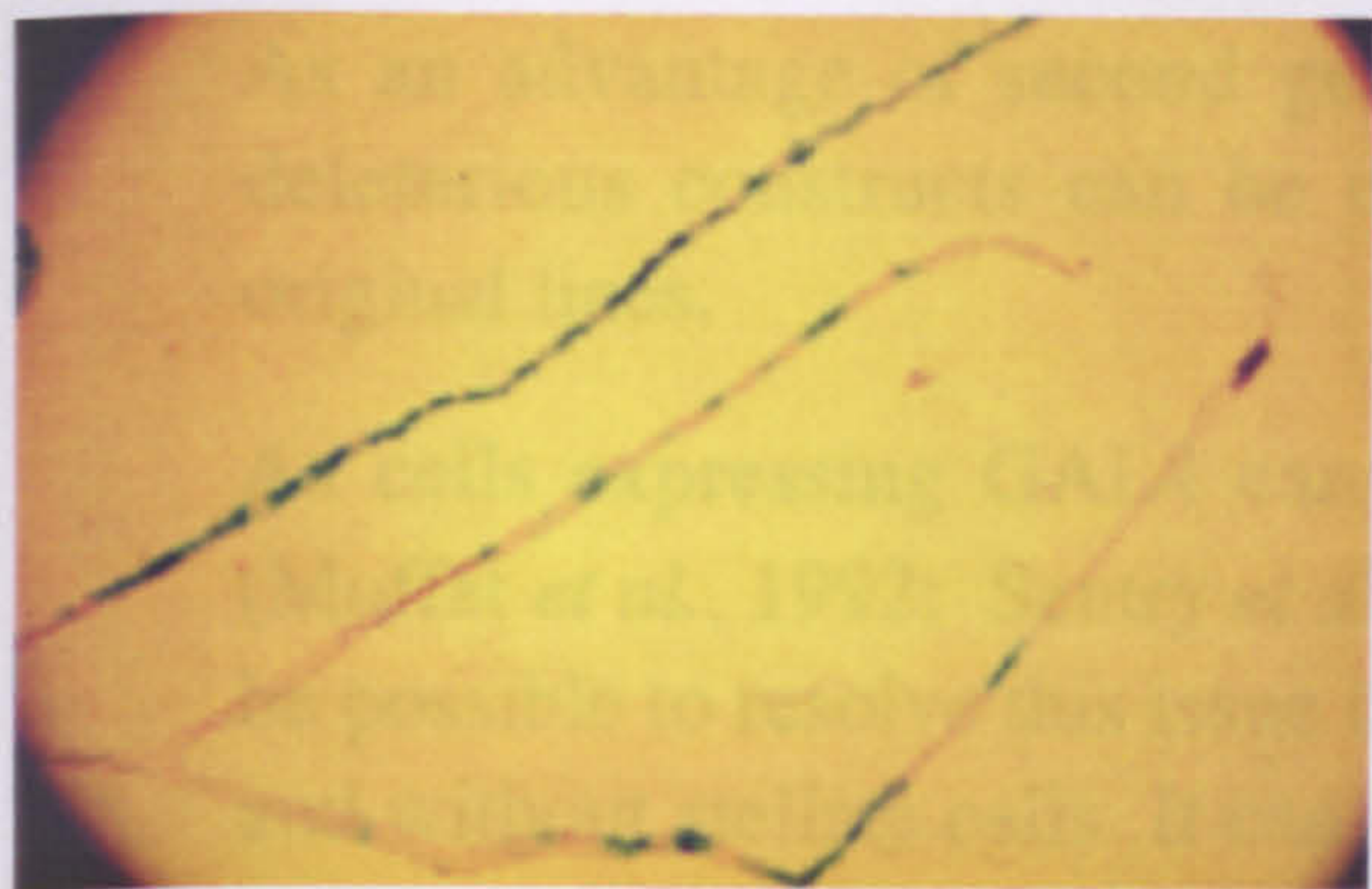


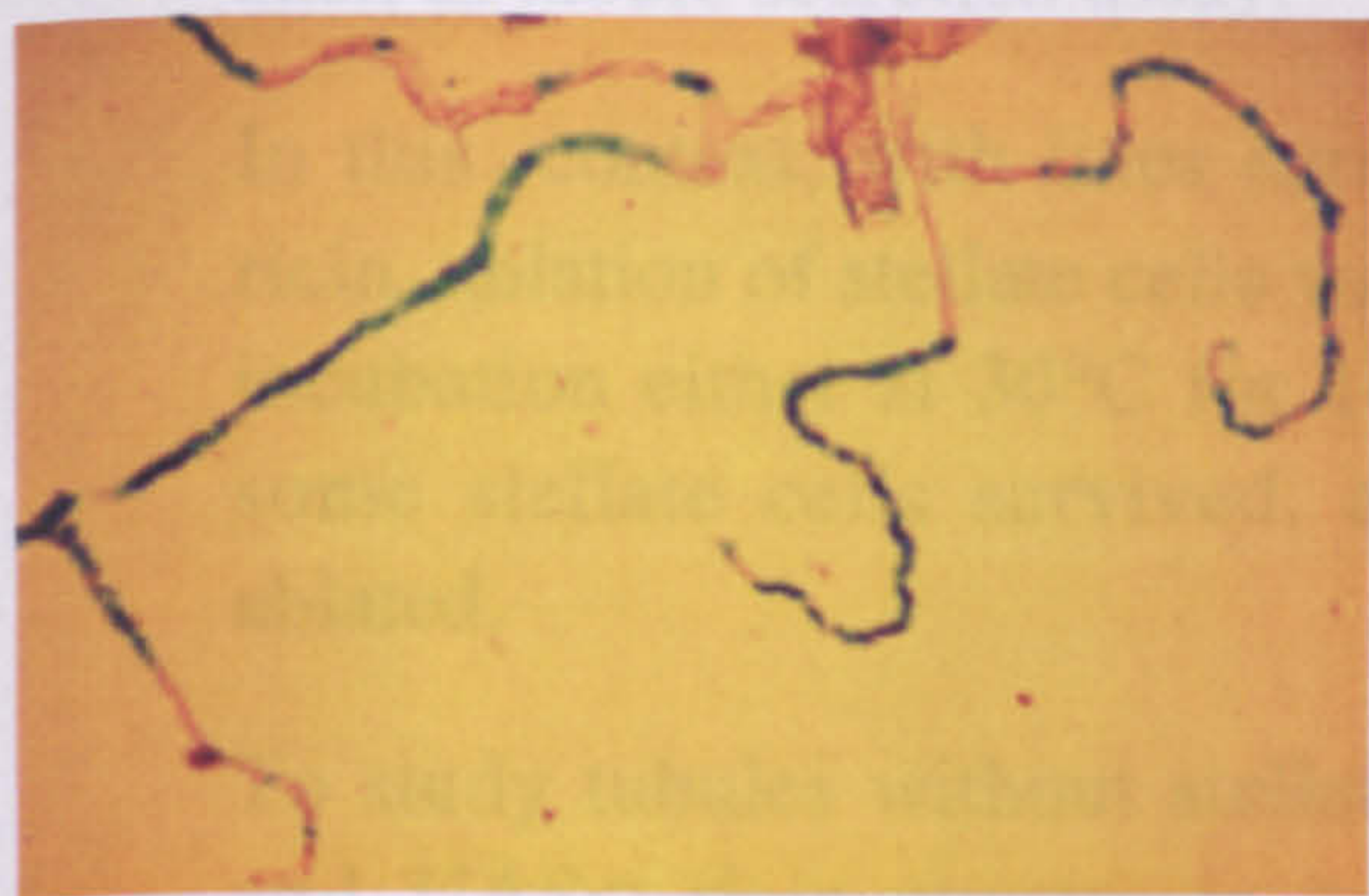
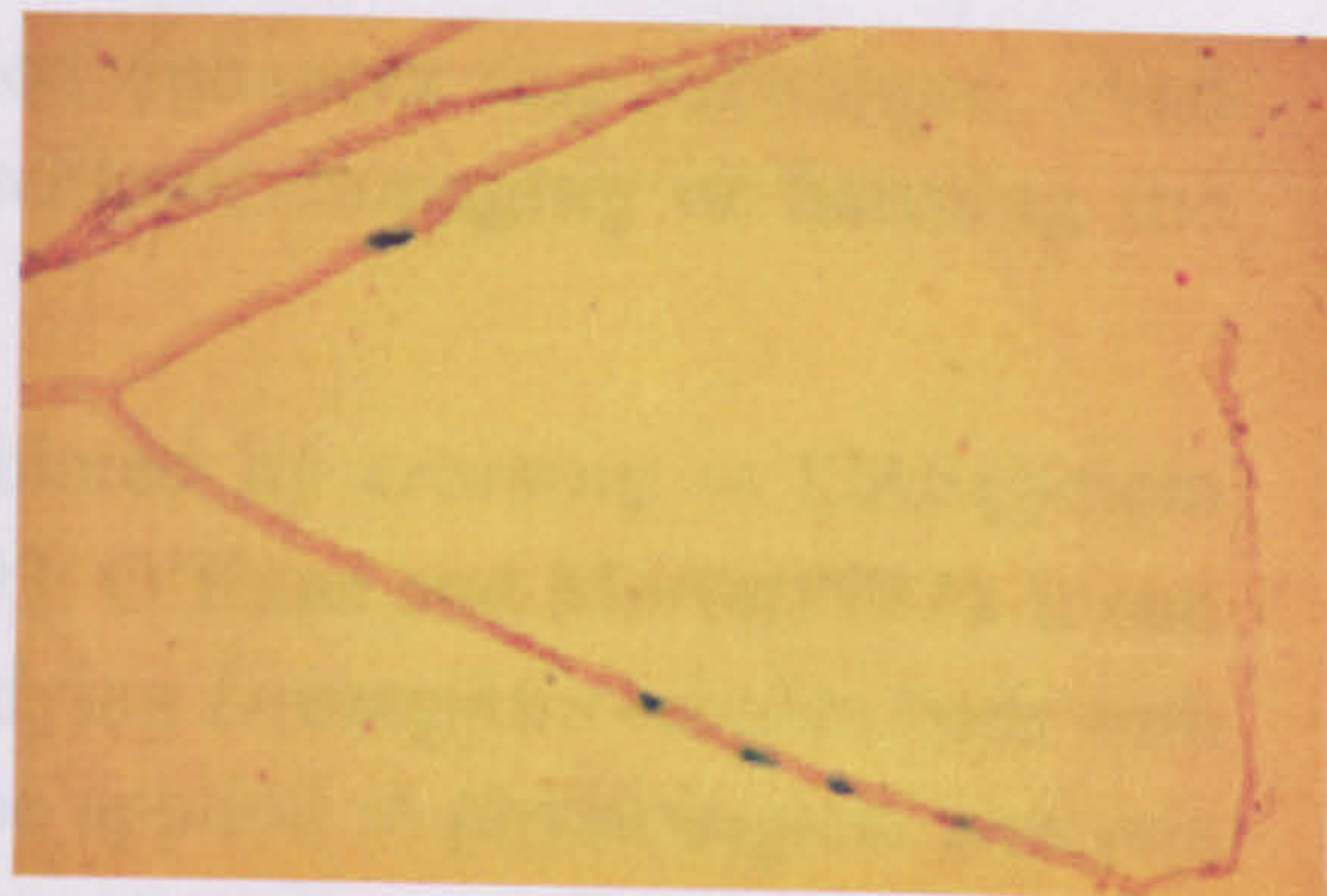
Figure 3.29. Ablation of stellate cells in GAL4 line, c710.

- a) Heat shocked stellate cells from anterior Malpighian tubule at 30°C for 10 days.
 - b) The view of non-heat-shocked stellate cells from anterior Malpighian tubule of control line.
 - c) A few stellate cells heat shocked from anterior Malpighian tubule.
 - d) Non heat-shocked control for (c).
 - e) Several heat shocked stellate cells probably partially ablated.
 - g) A single ablated (partially) stellate cell.
 - h) A single non-ablated stellate cell.
-

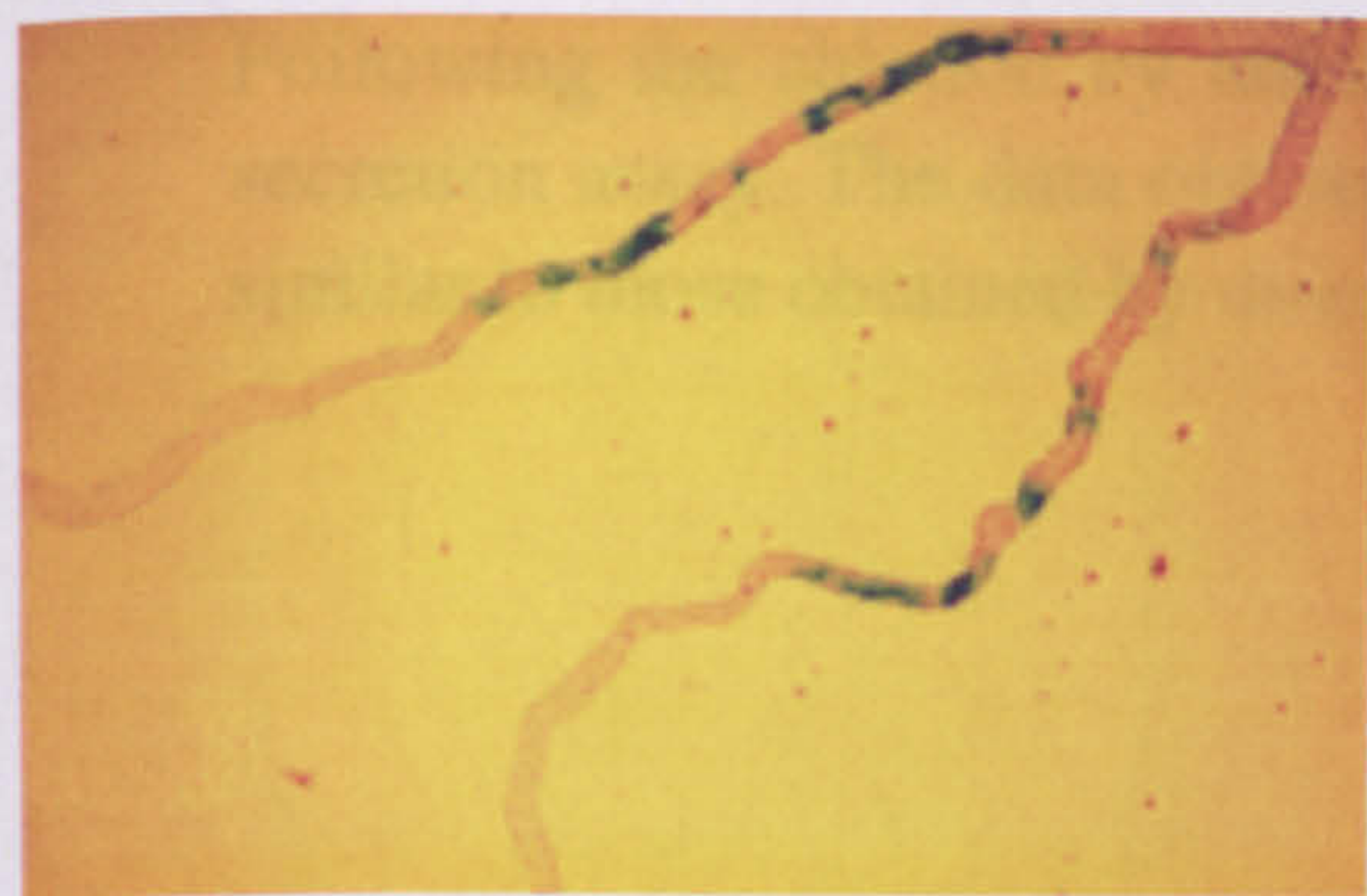
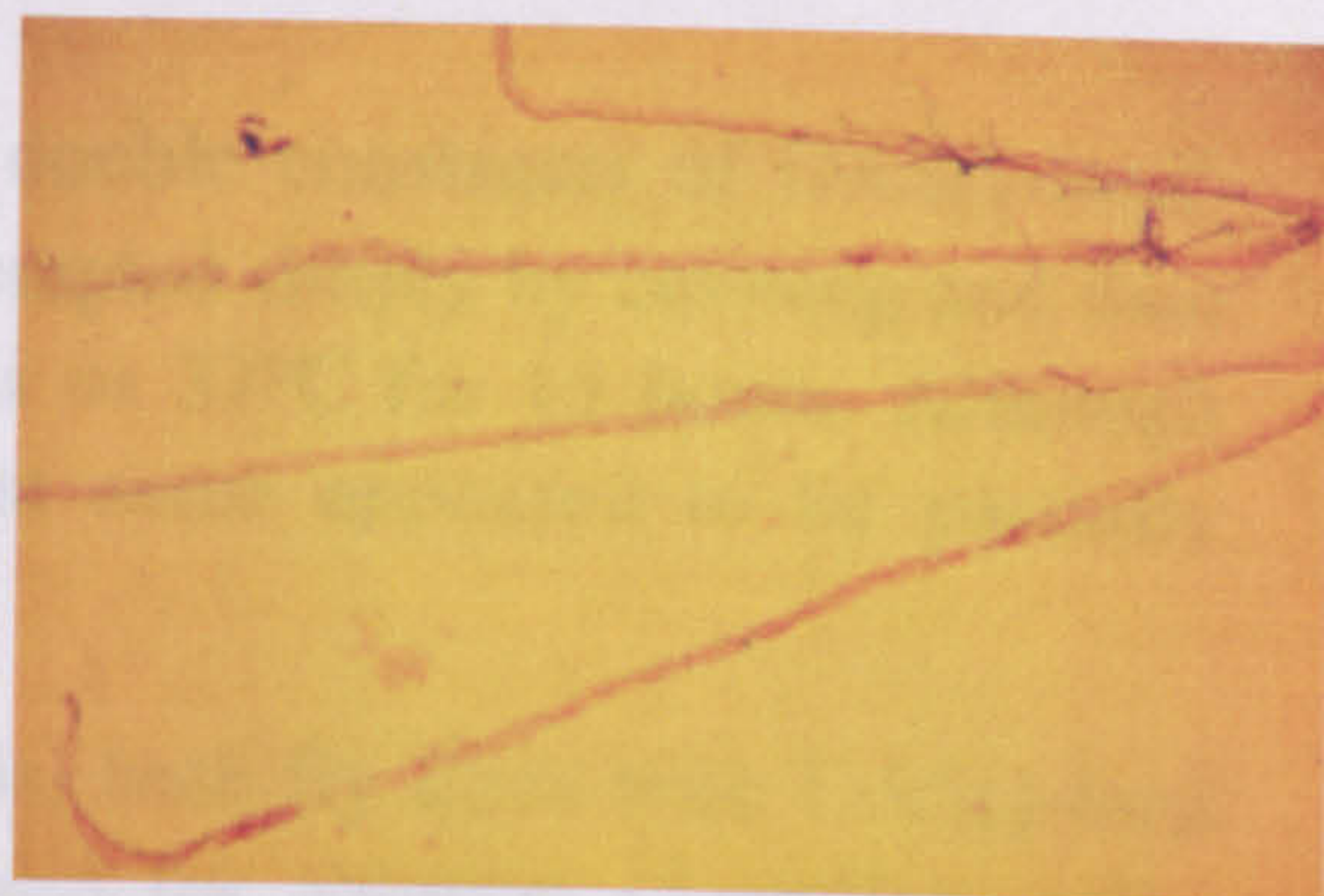
3.7.3. Ablation of stellate cells



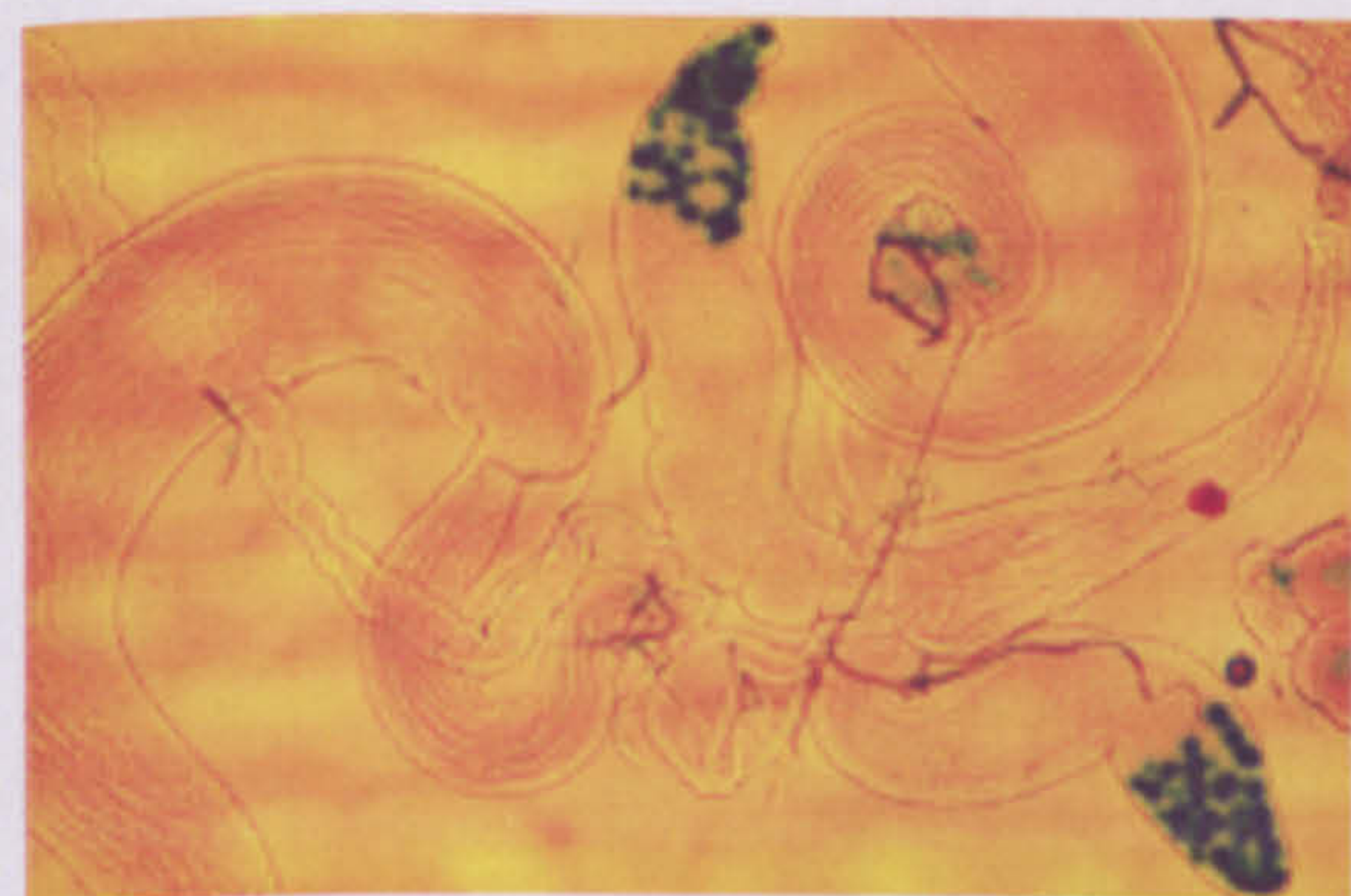
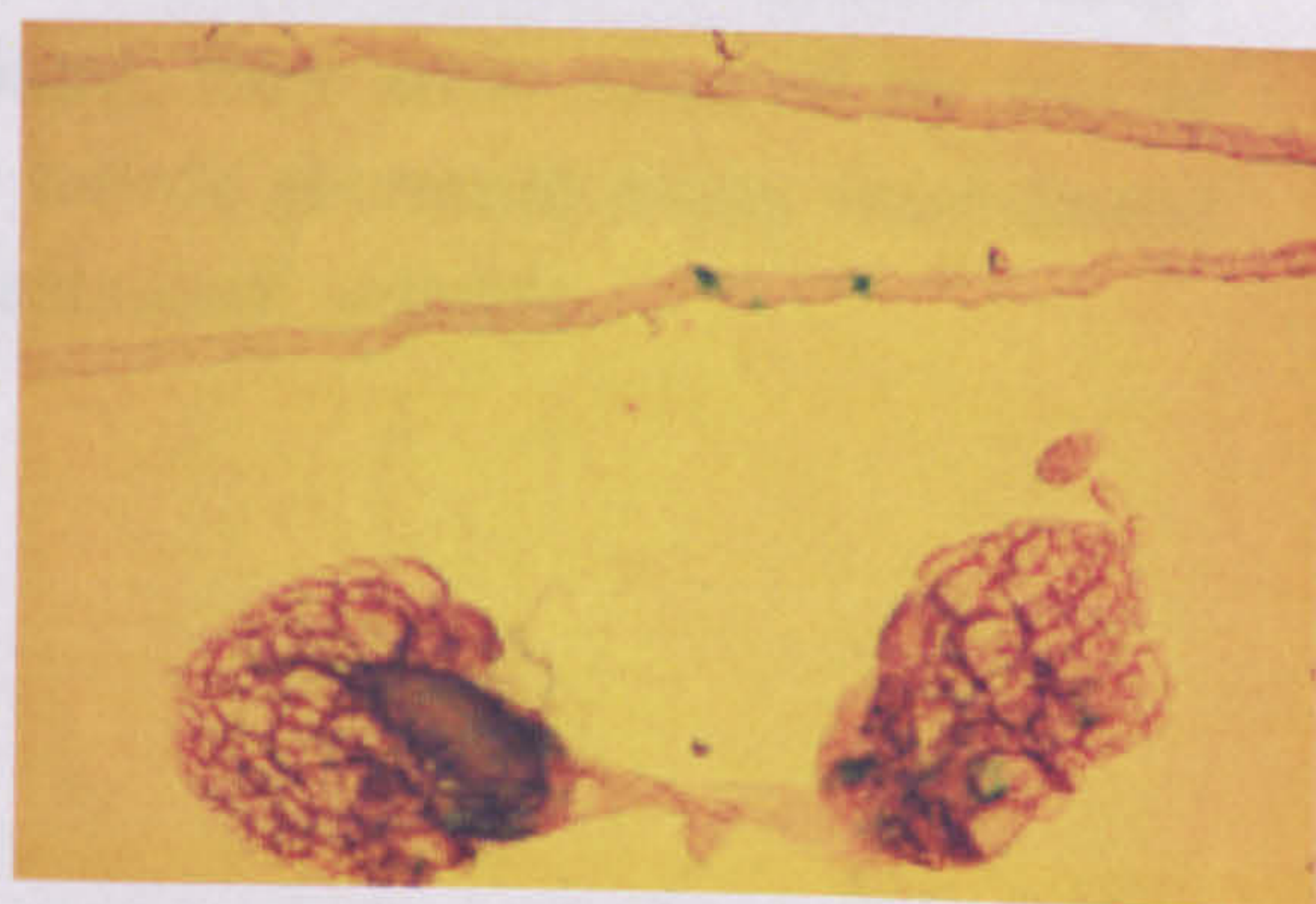
b)



d)



f)



h)



3.7.3. Ablation of stellate cells

As an advantage of second generation enhancer trap technique, even deleterious constructs can be used without damaging or harming the original lines.

As cells expressing GAL4 can be ablated by crossing to UASG-ricin (Moffat *et al.*, 1992; Sentry *et al.*, 1993; O’Kane and Moffat 1992), it can be possible to resolve this issue by studying *Drosophila* tubules both with and without stellate cells. It can also be applied to physiological analysis such as tubule secretion assay.

In this context, with lines carrying double constructs of UASG-*lacZ*-ricin, ablation of stellate cells was attempted simply by crossing and then incubation either at 30°C for 10 days or 37°C for 15 hours. Although some stellate cells survived, at least some appeared to be partially ablated.

To study tubules without stellate cells, both fly lines with UASG-*lacZ* and UASG-ricin constructs were used for ablation of stellate cells. Following the ablation by heat-shocking, they were assayed by tubule secretion assay. The data obtained by tubule secretion assay were found similar to those obtained by direct expressional activity.

Figure 3.28. Attempted ablation of stellate cells from GAL4 line, c724, crossing with the flies carrying UAS_G-ricin and UAS_G-*lacZ* constructs.

- a) Some anterior Malpighian tubule stellate cells, heat shocked at 30°C for 10 days.
 - b) A control line that was not heat shocked.
 - c) A few stellate cells, after an ablation attempt by heat shocking, at higher magnification.
 - d) A view of anterior Malpighian tubule which was not heat shocked as a control.
 - e) Some heat shocked stellate cells from posterior Malpighian tubule.
 - f) Control for the previous figure.
 - g) Two of the heat shocked stellate cells.
 - h) Control line which was not heat shocked.
-

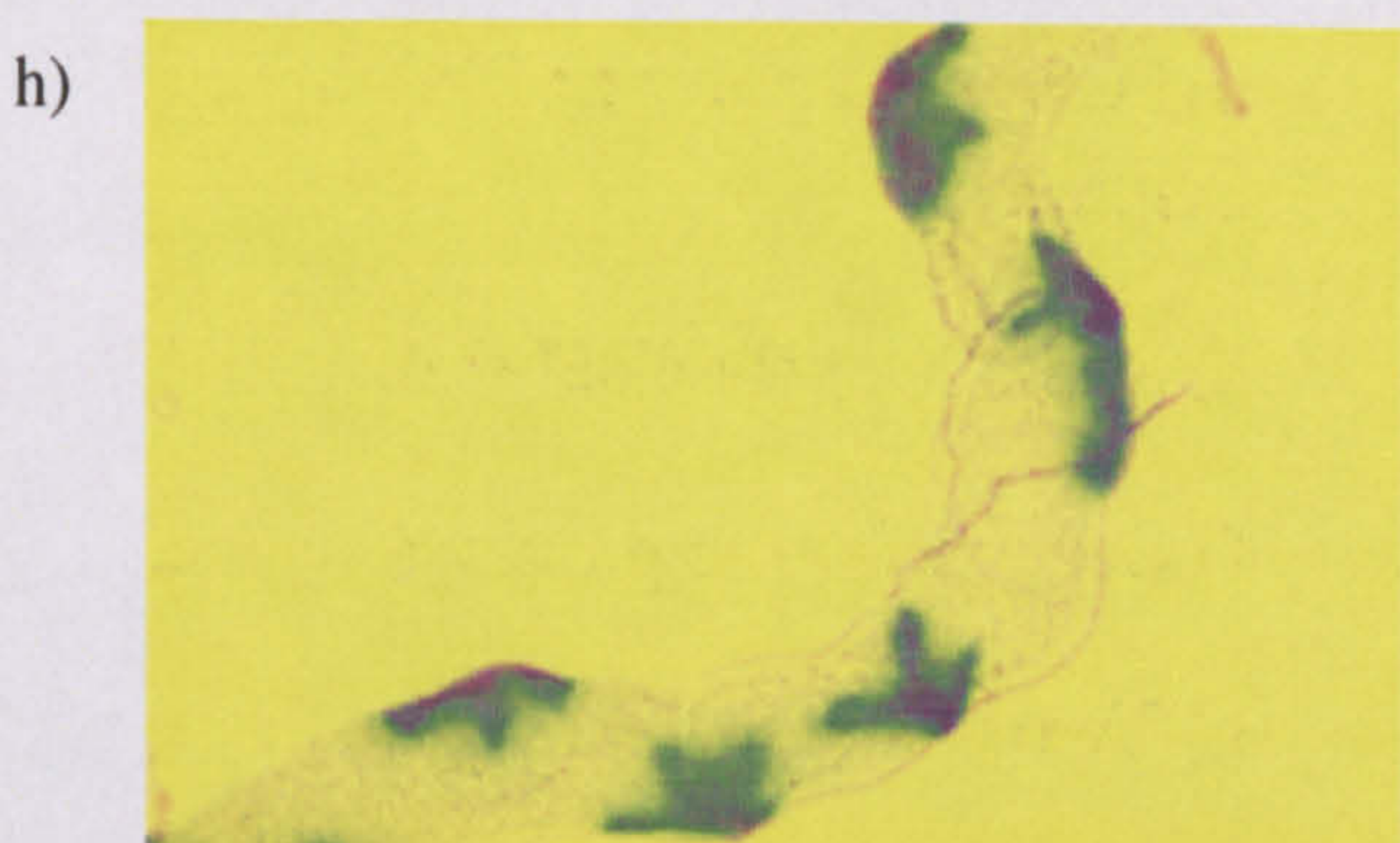
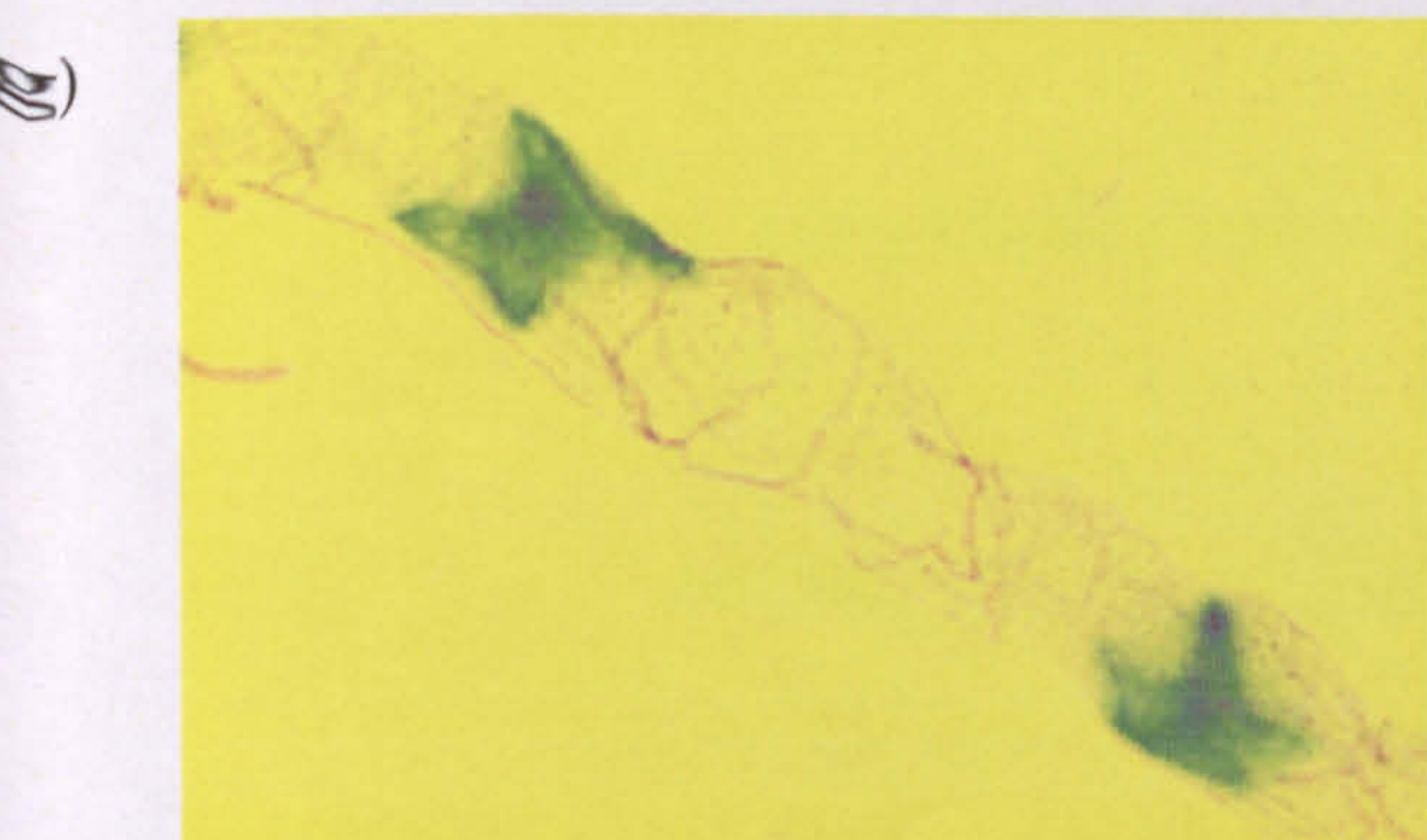
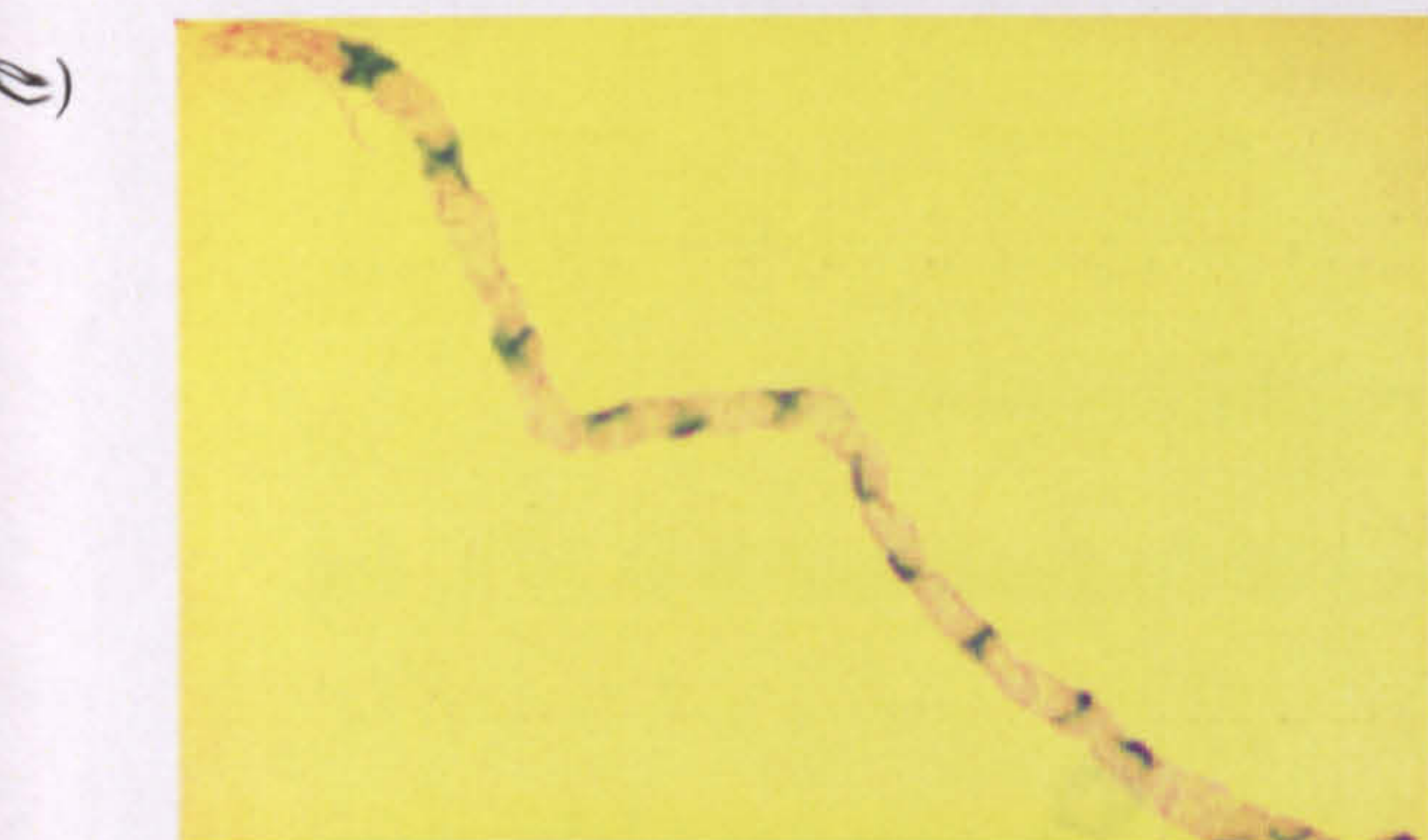
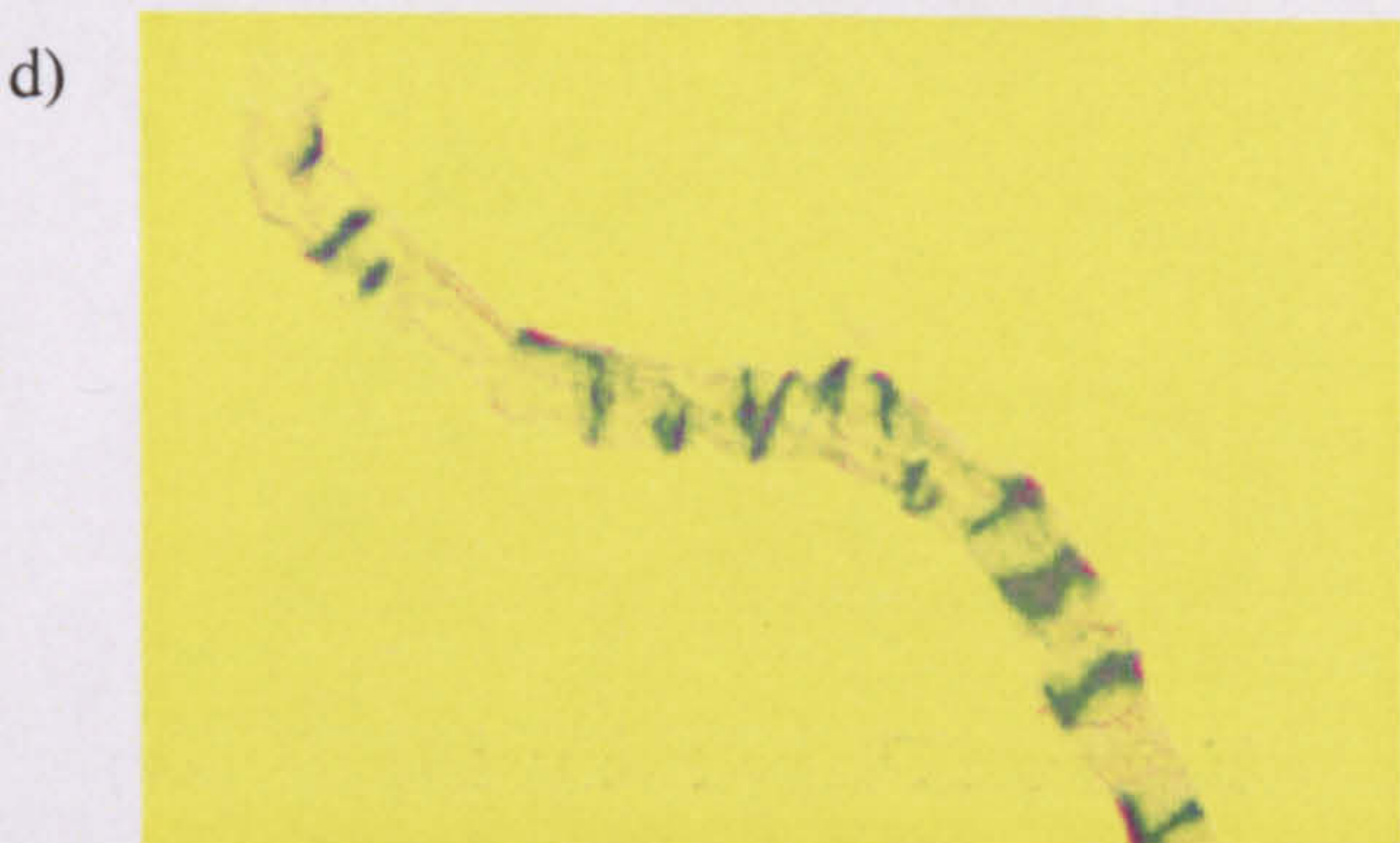
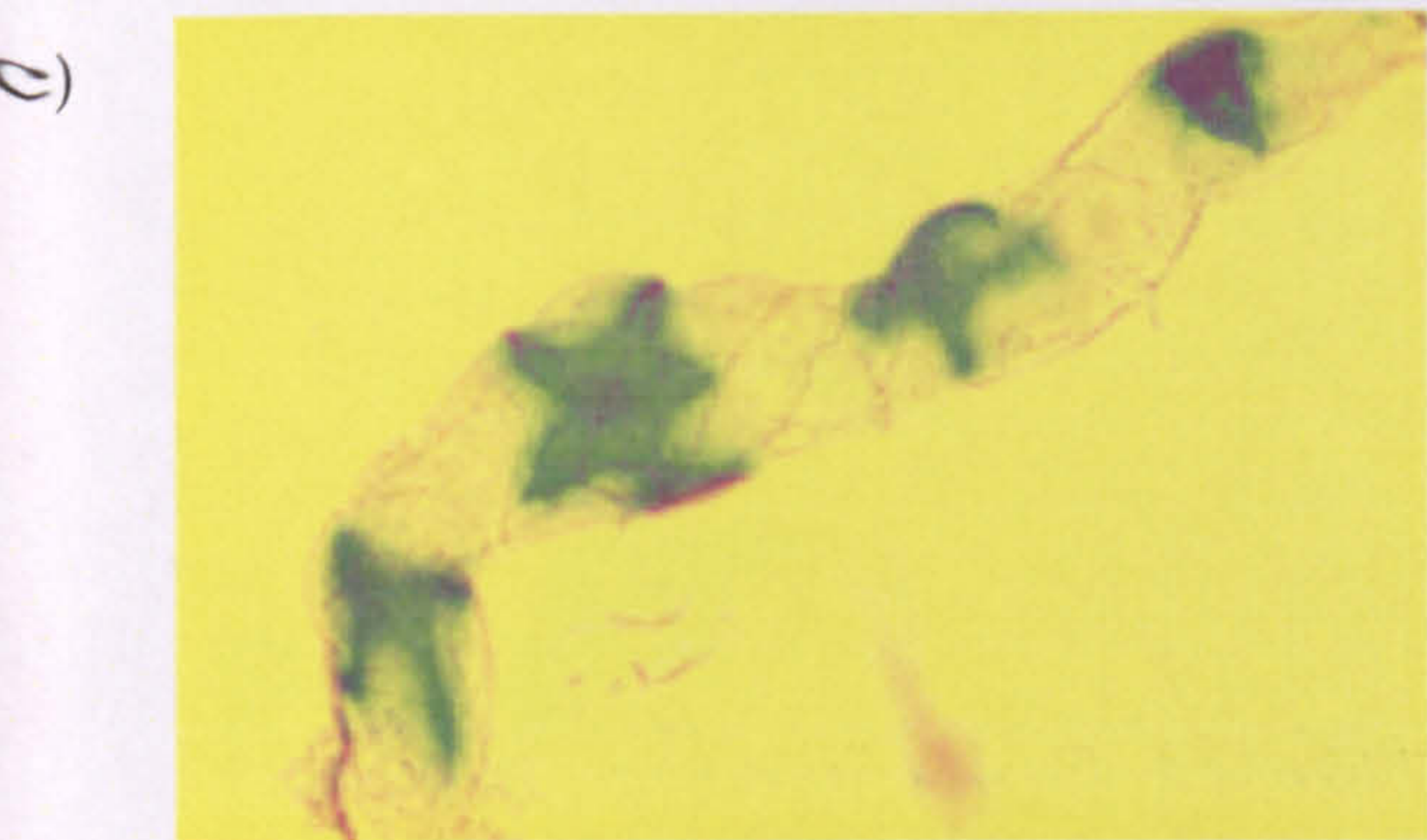
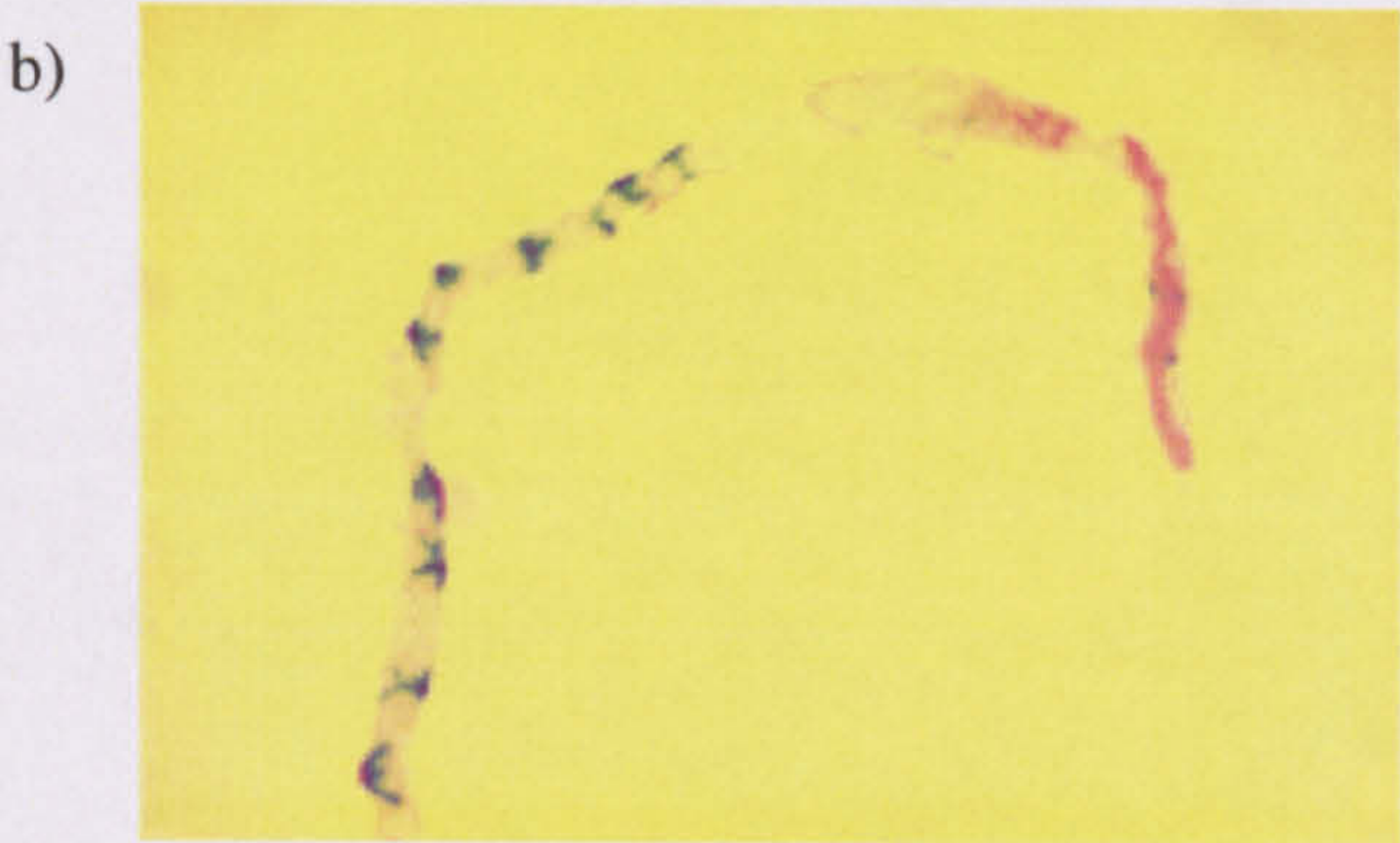
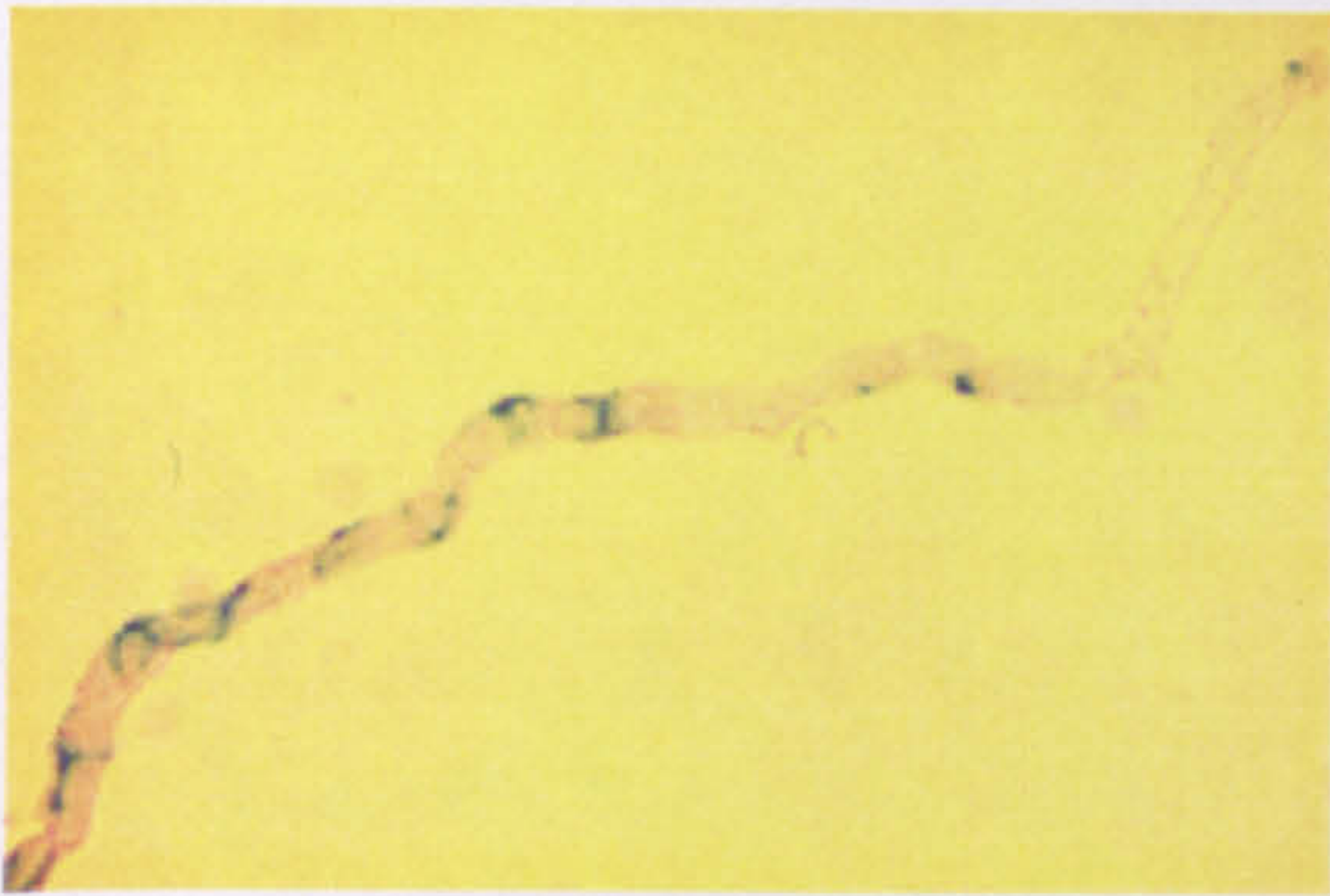


Figure 3.29. Ablation of stellate cells in GAL4 line, c710.

- a) Heat shocked stellate cells from anterior Malpighian tubule at 30°C for 10 days.
 - b) The view of non-heat-shocked stellate cells from anterior Malpighian tubule of control line.
 - c) A few stellate cells heat shocked from anterior Malpighian tubule.
 - d) Non heat-shocked control for (c).
 - e) Several heat shocked stellate cells probably partially ablated.
 - g) A single ablated (partially) stellate cell.
 - h) A single non-ablated stellate cell.
-

a)



b)



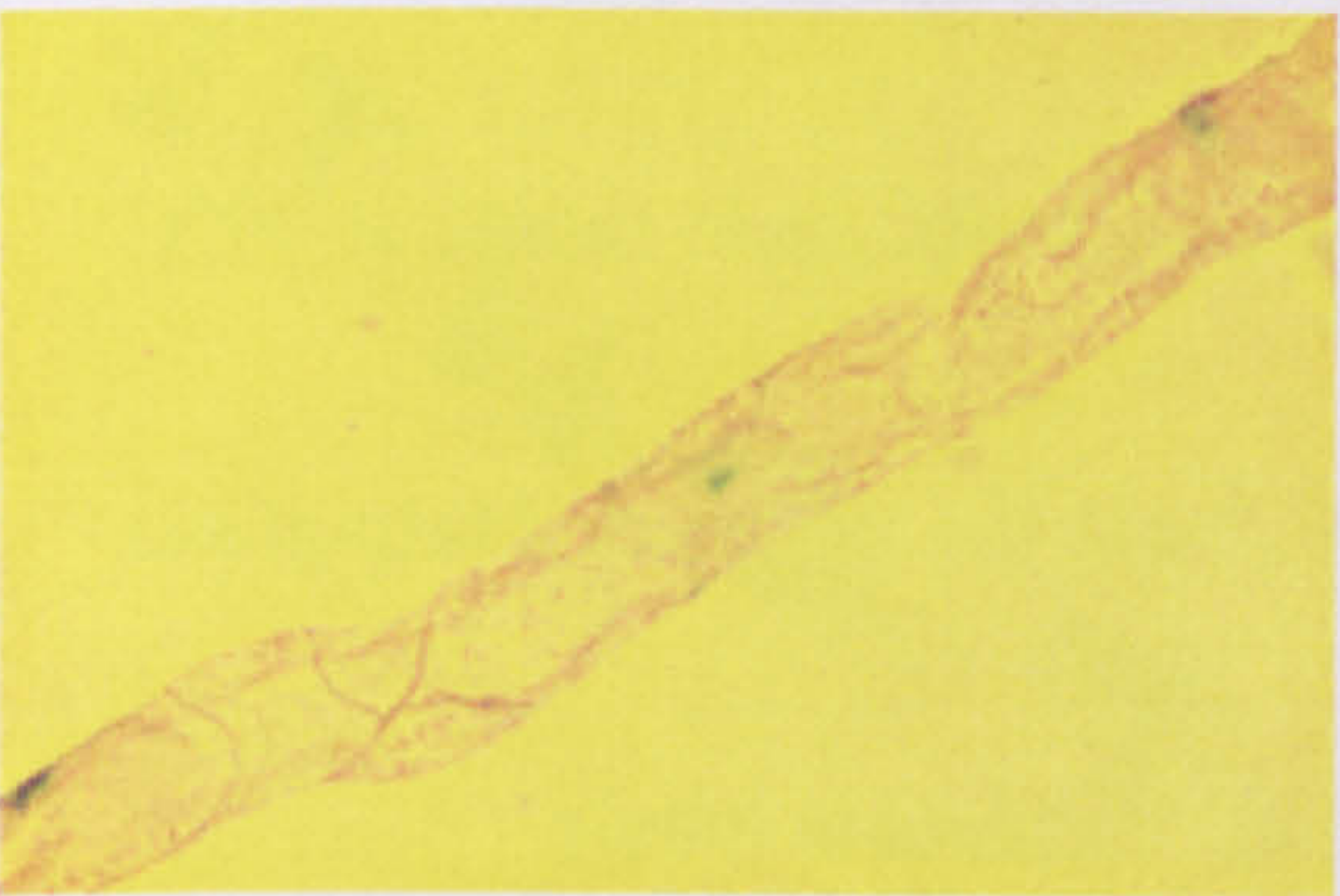
c)



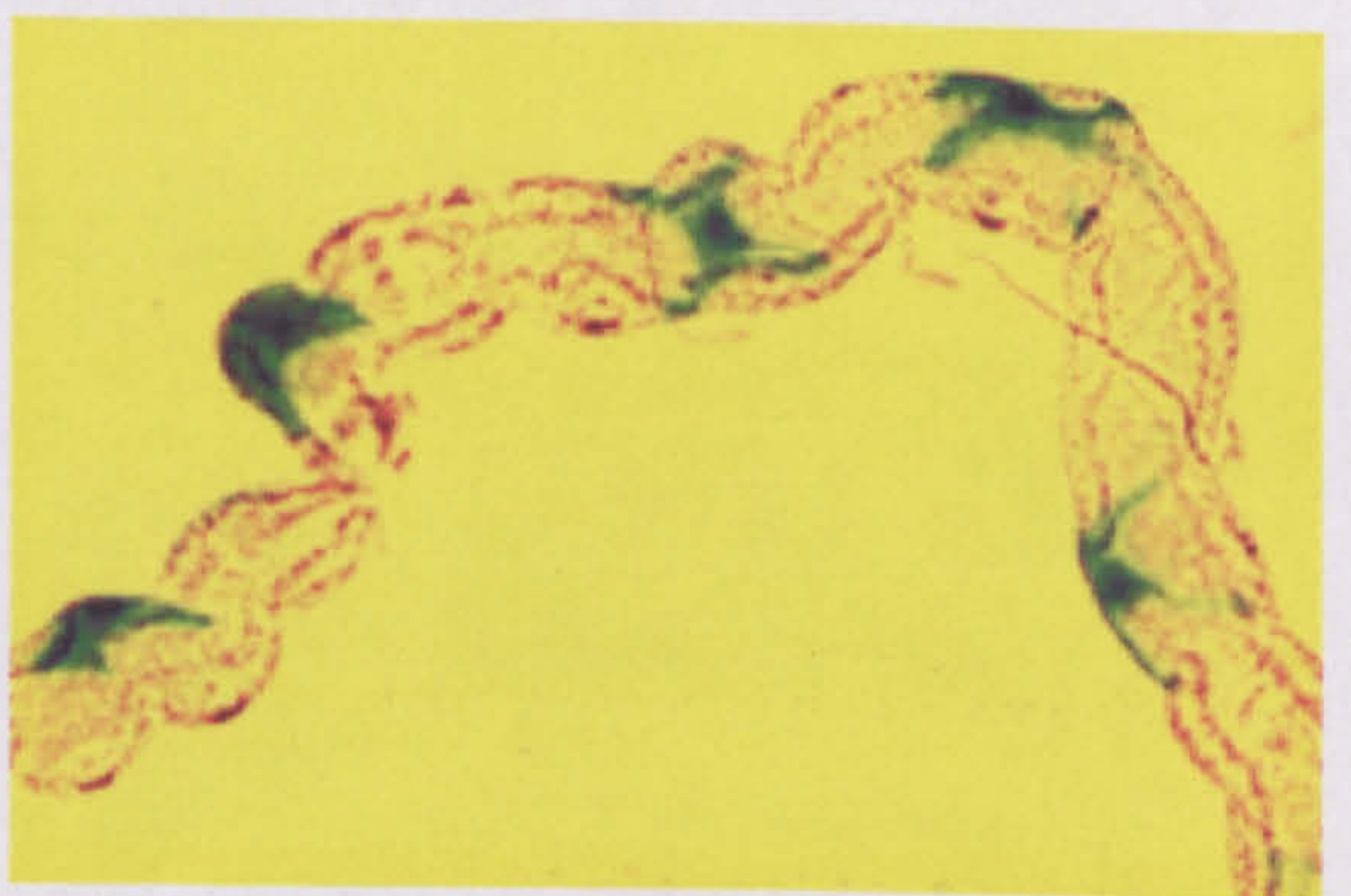
d)



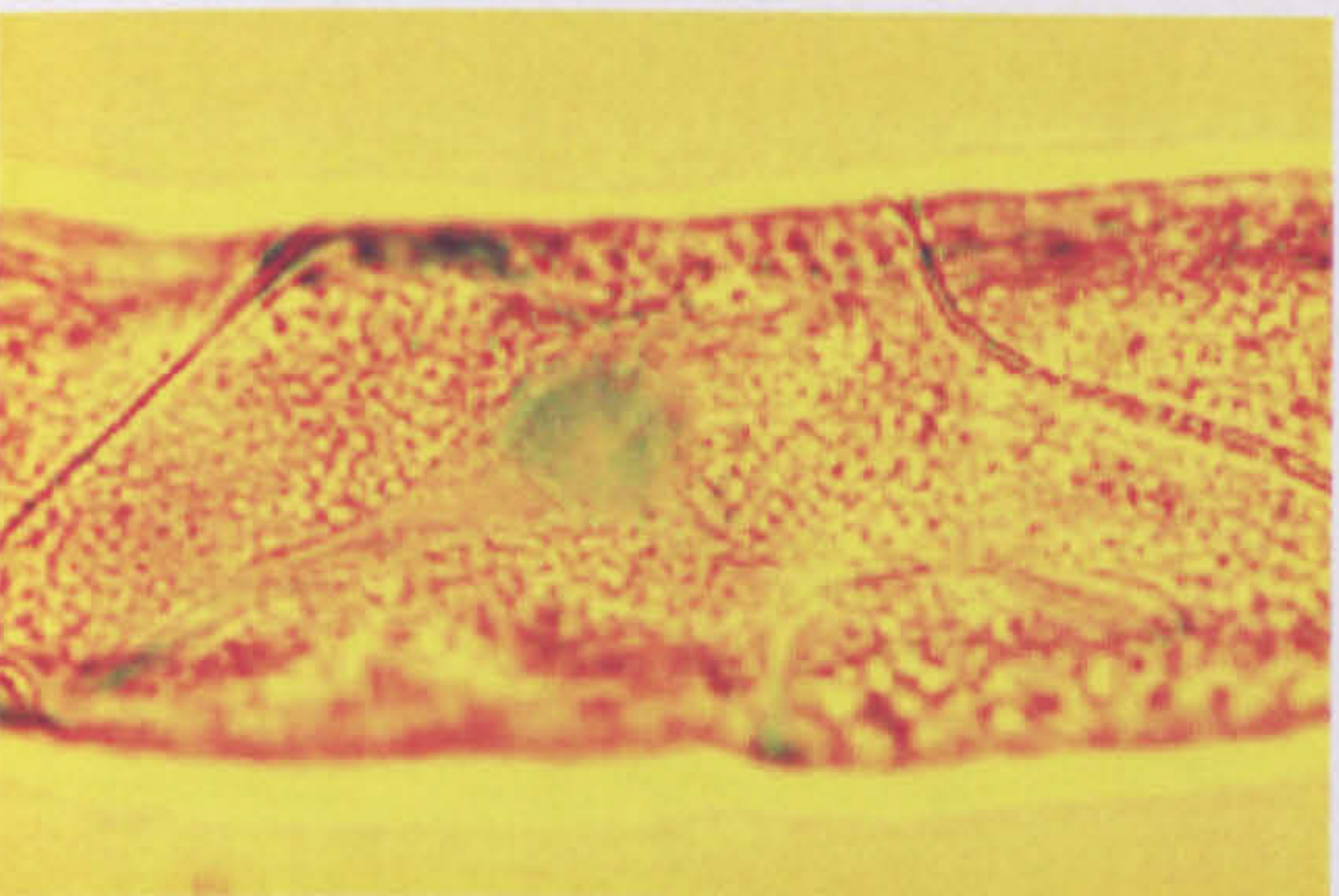
e)



f)



g)



h)

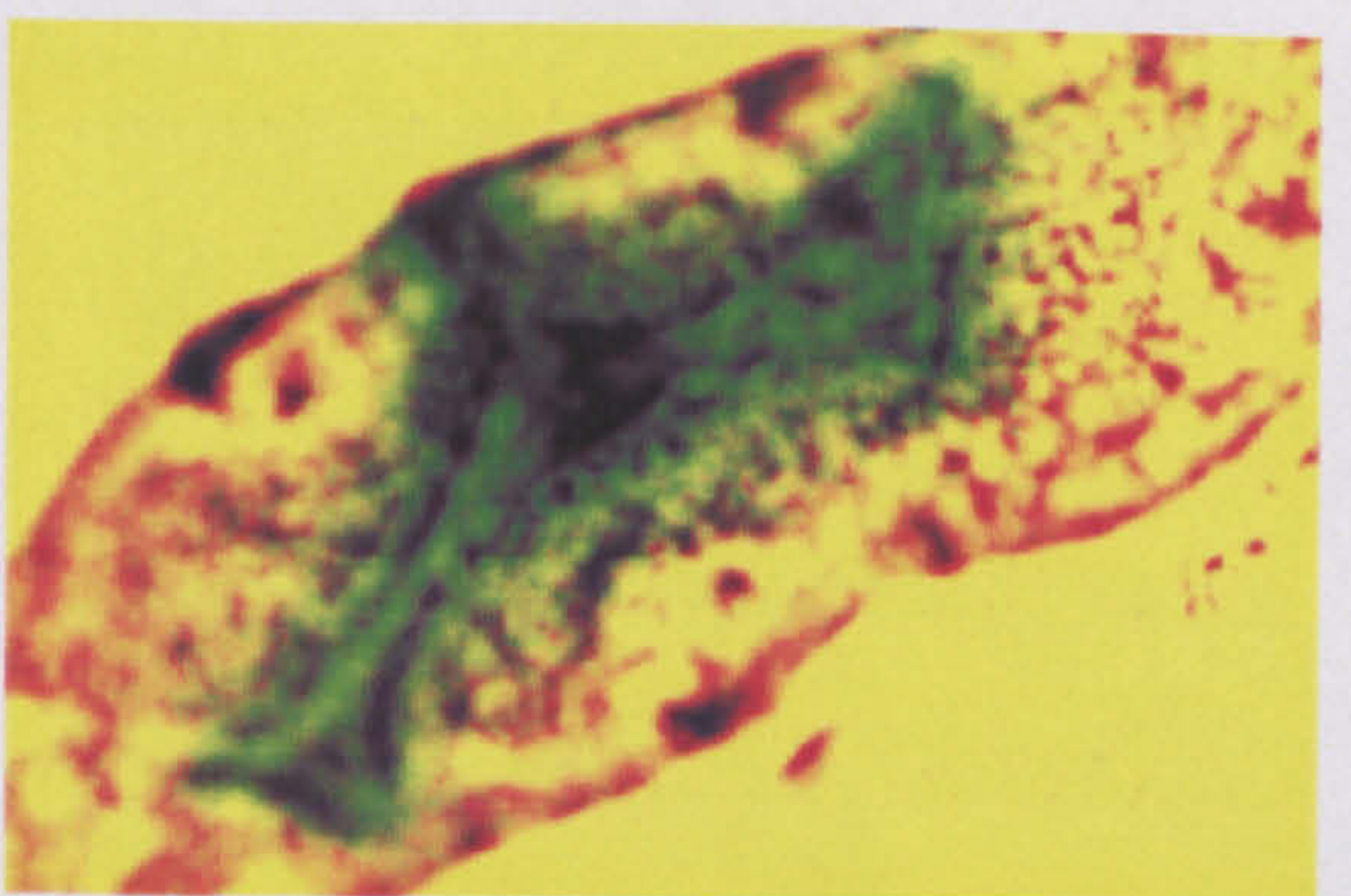


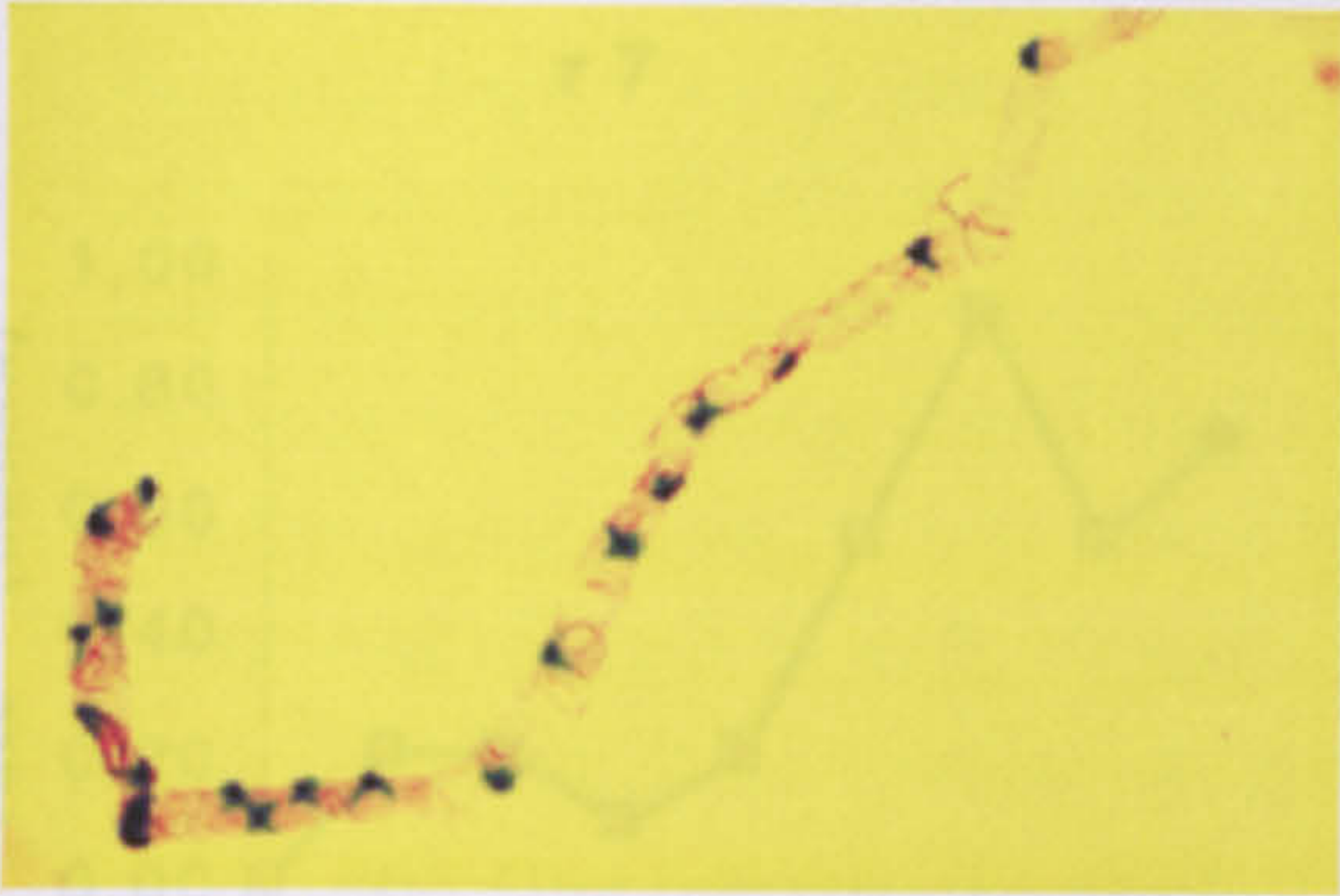
Figure 3.30. Ablation of stellate cells from line c724 at 37°C for 24 hours.

- a) Heat shocked stellate cells from posterior Malpighian tubule at 30°C for 10 days..
 - b) Two non-heat-shocked stellate cells from the posterior Malpighian tubules of a control line kept at 18°C.
 - c) Heat shocked stellate cells from anterior Malpighian tubule.
 - d) A few stellate cells heat shocked from anterior Malpighian tubule.
 - e) Several heat shocked stellate cells from anterior Malpighian tubule.
 - f) Several non-heat-shocked stellate cells from posterior Malpighian tubule of a control line kept at 18°C.
 - g) Stellate cells of anterior Malpighian tubule heat shocked at 37°C for 15 hours from c724-GFP line crossed with URcs.21.
 - h) Non-heat-shocked stellate cells from posterior Malpighian tubule from control line kept at 18°C.
-

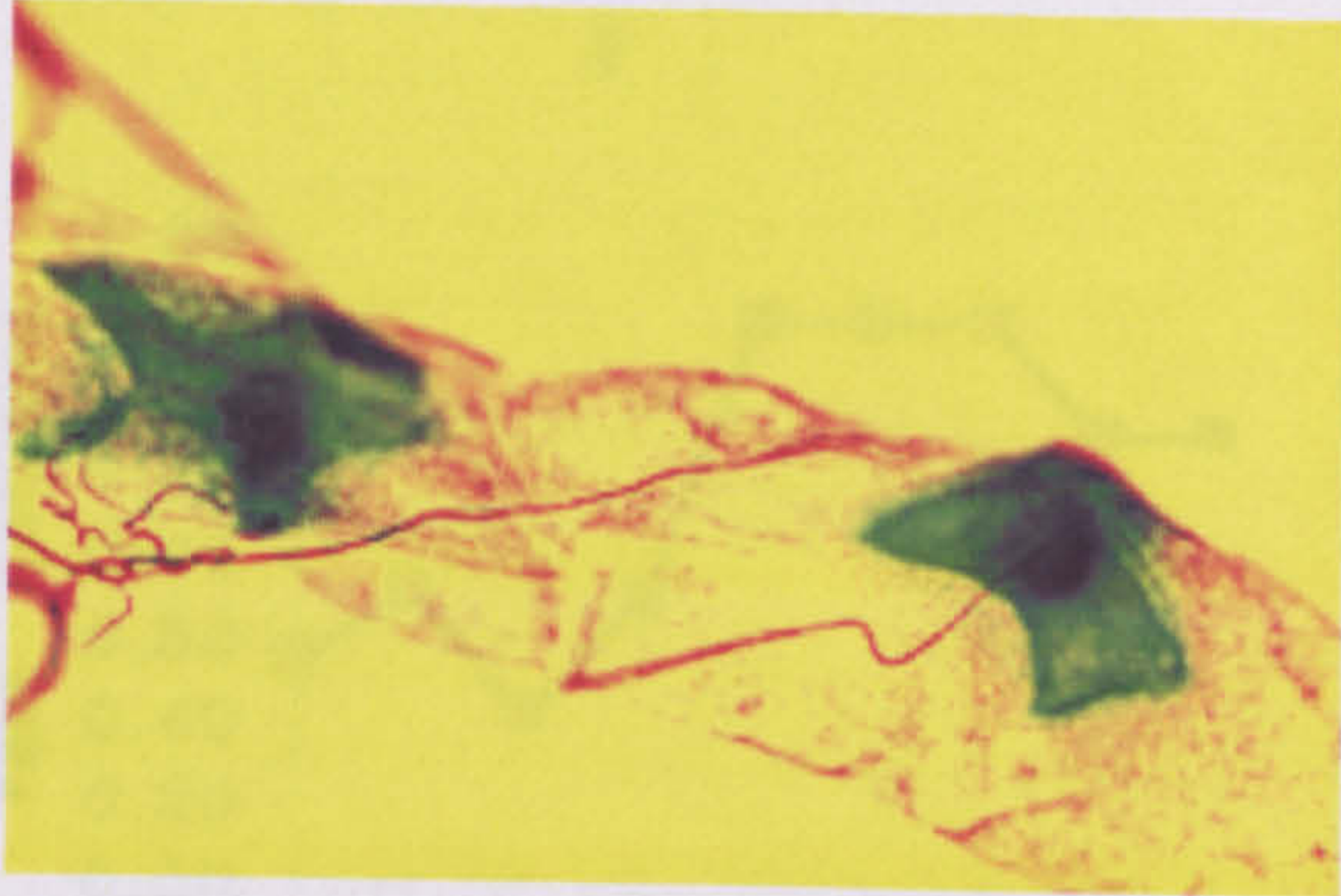
Line C724.

(a) Oregon-R

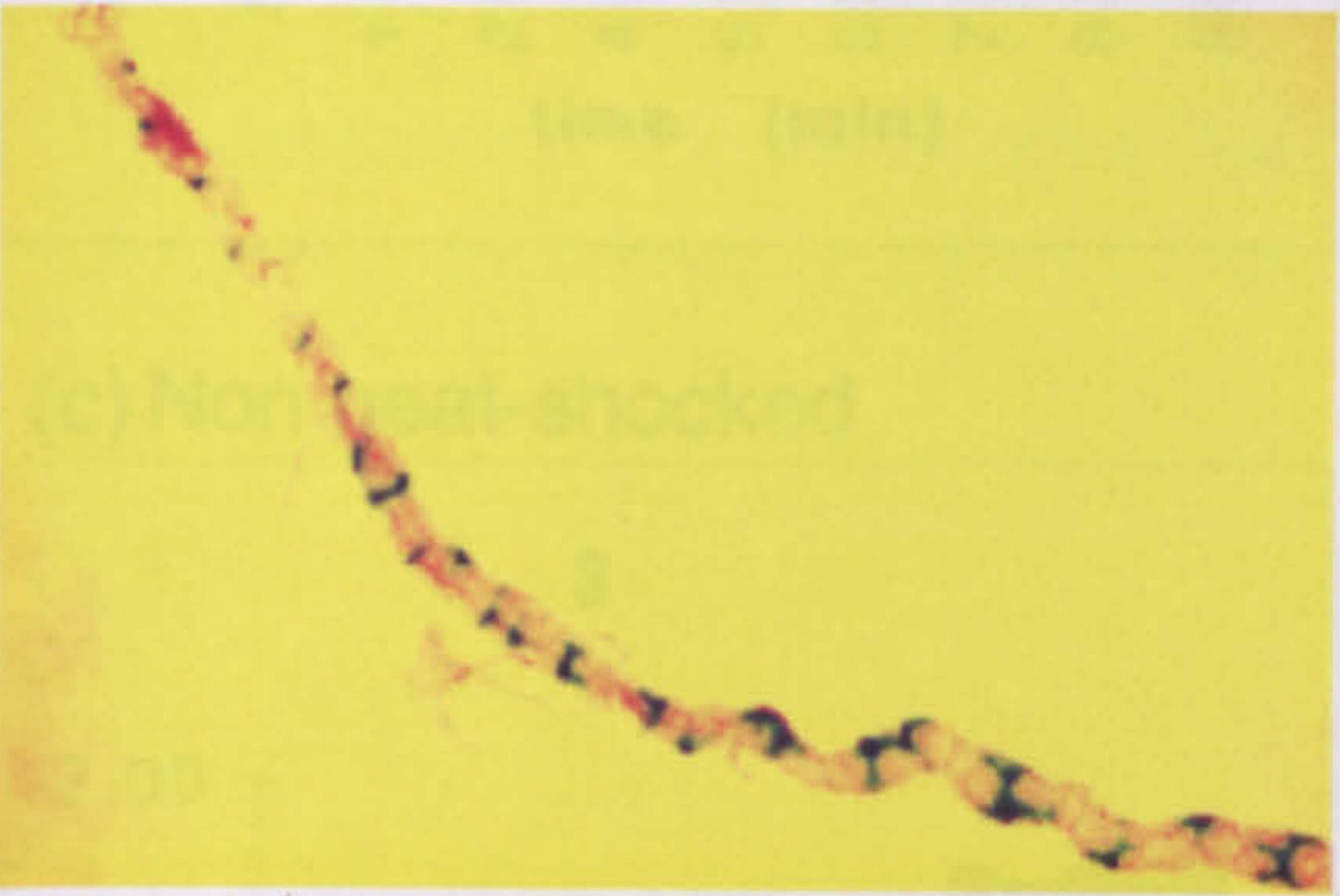
a)



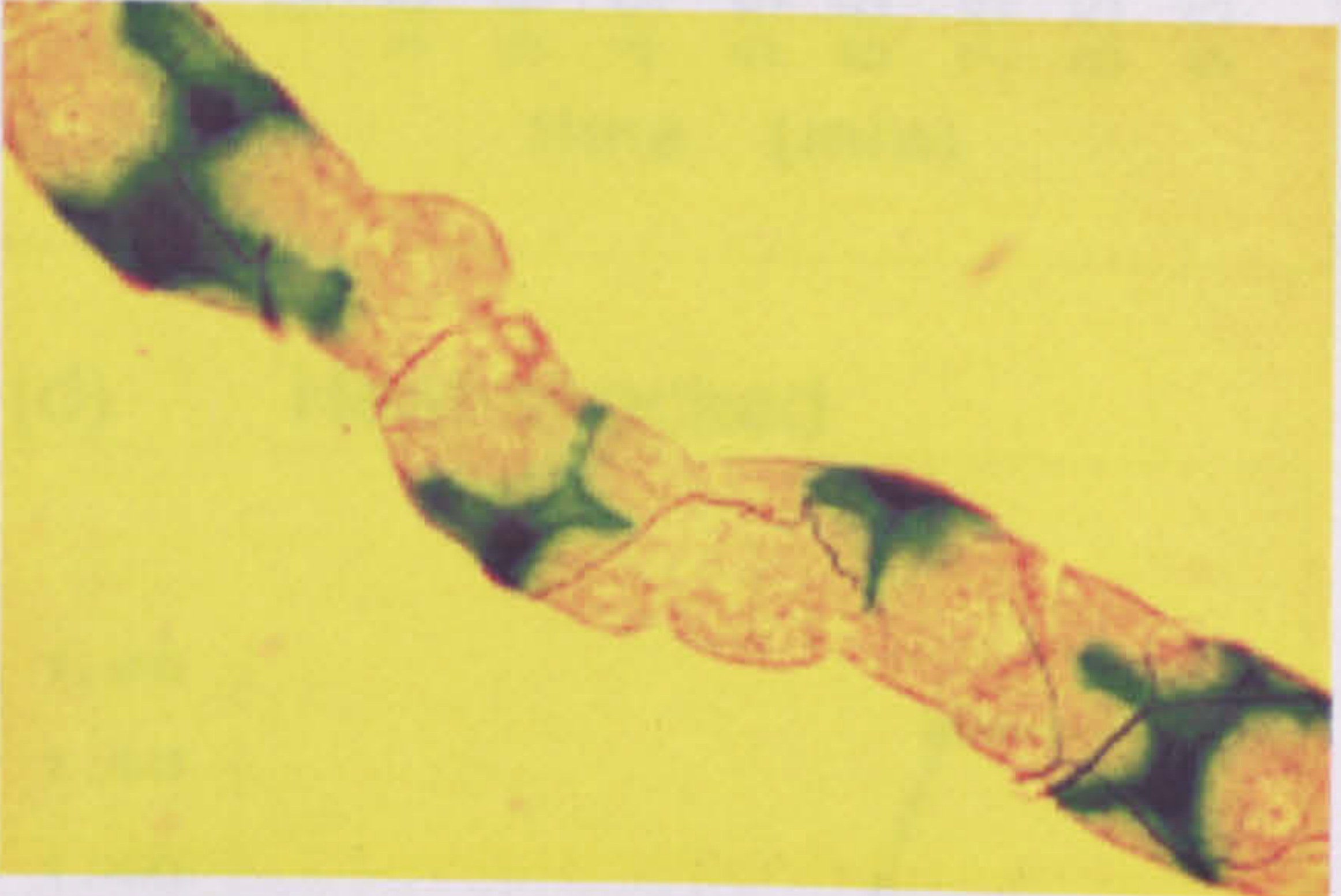
b)



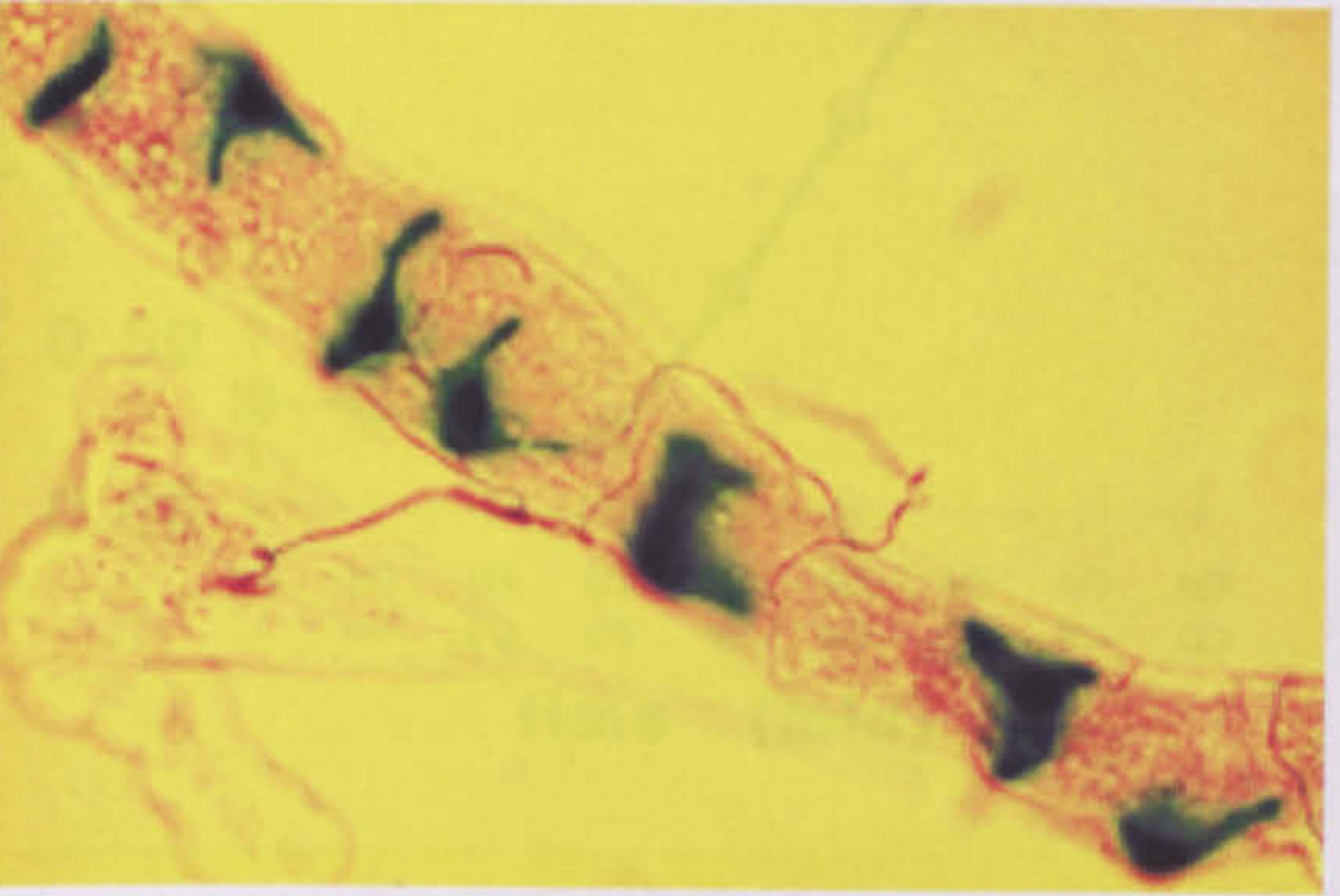
c)



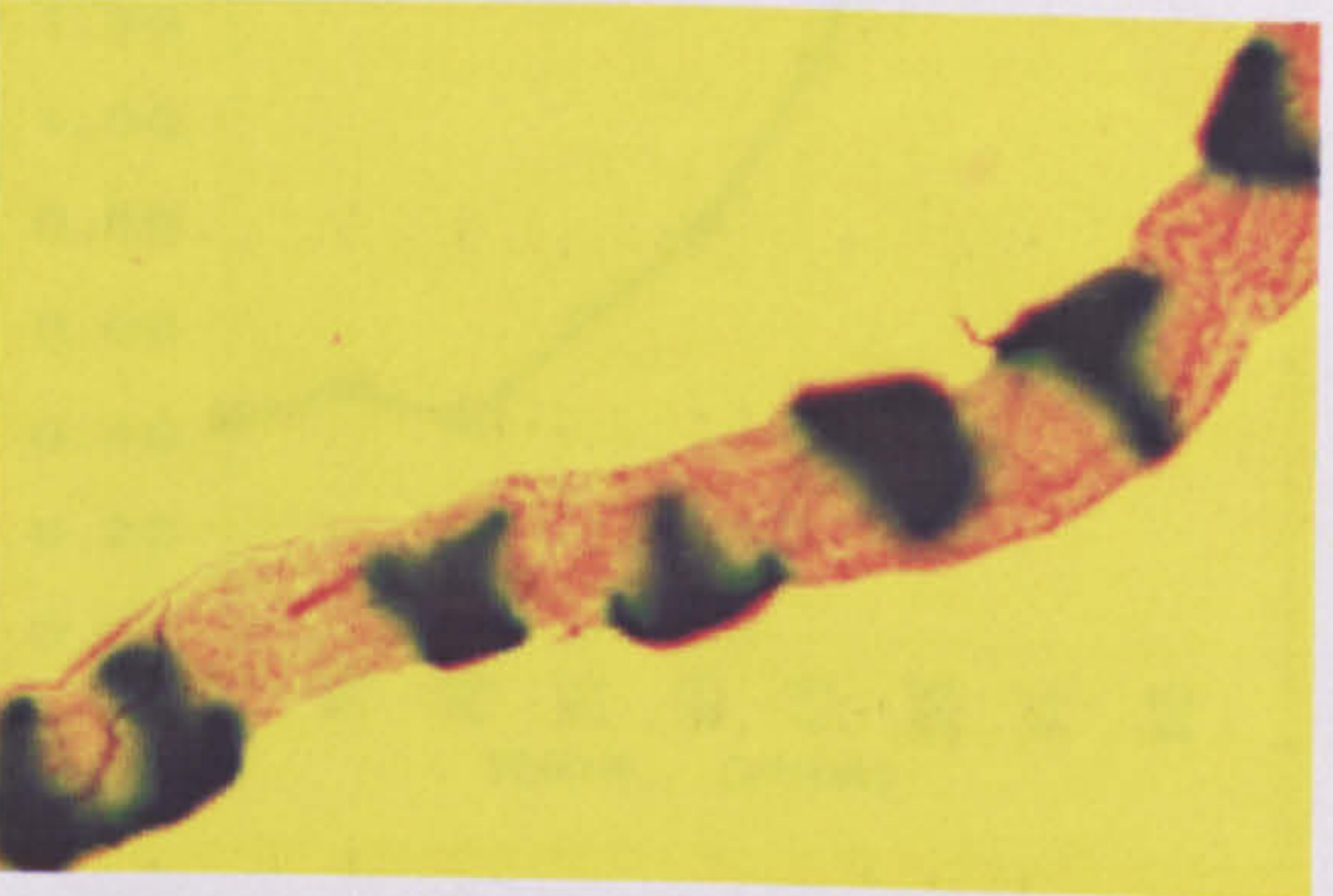
d)



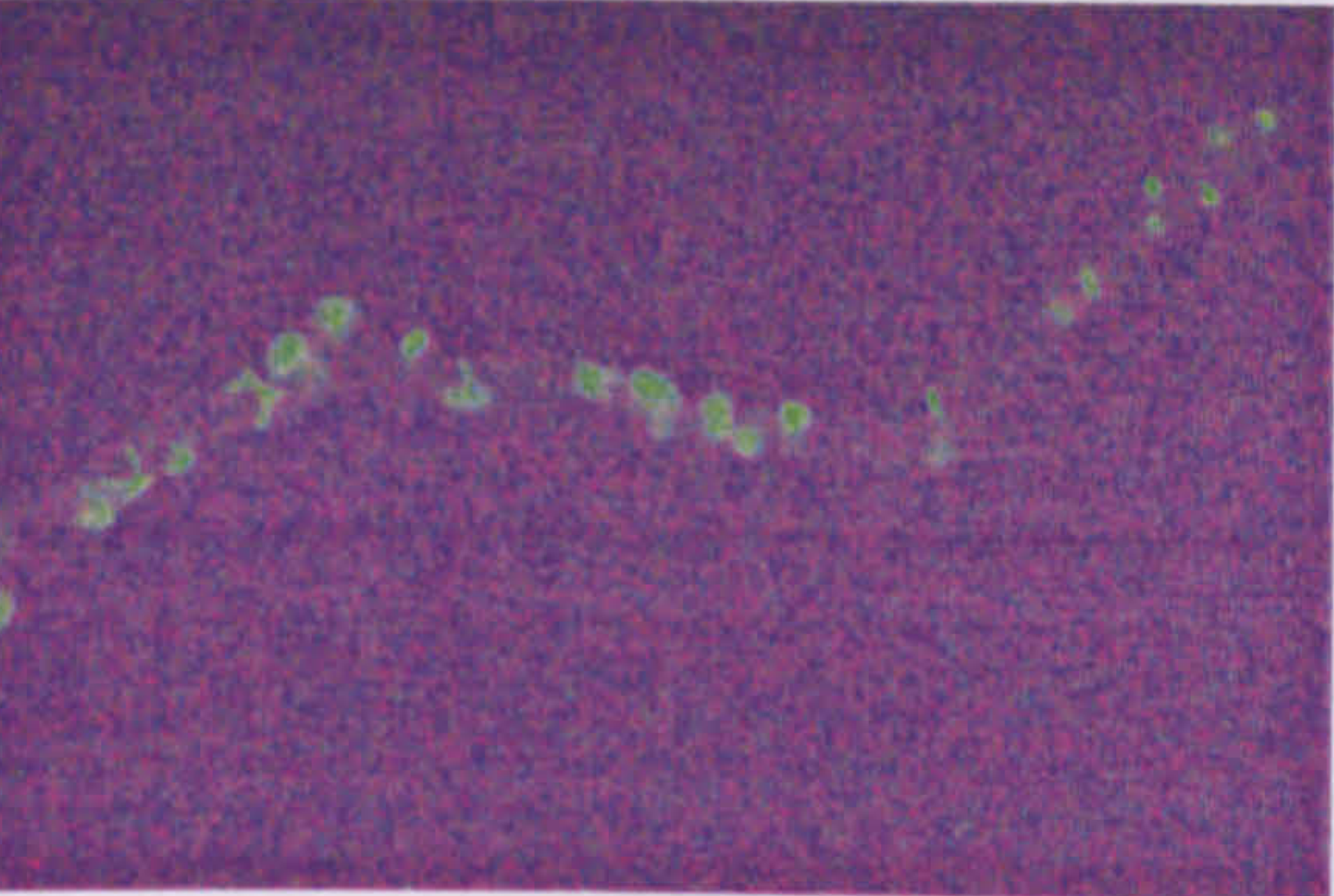
e)



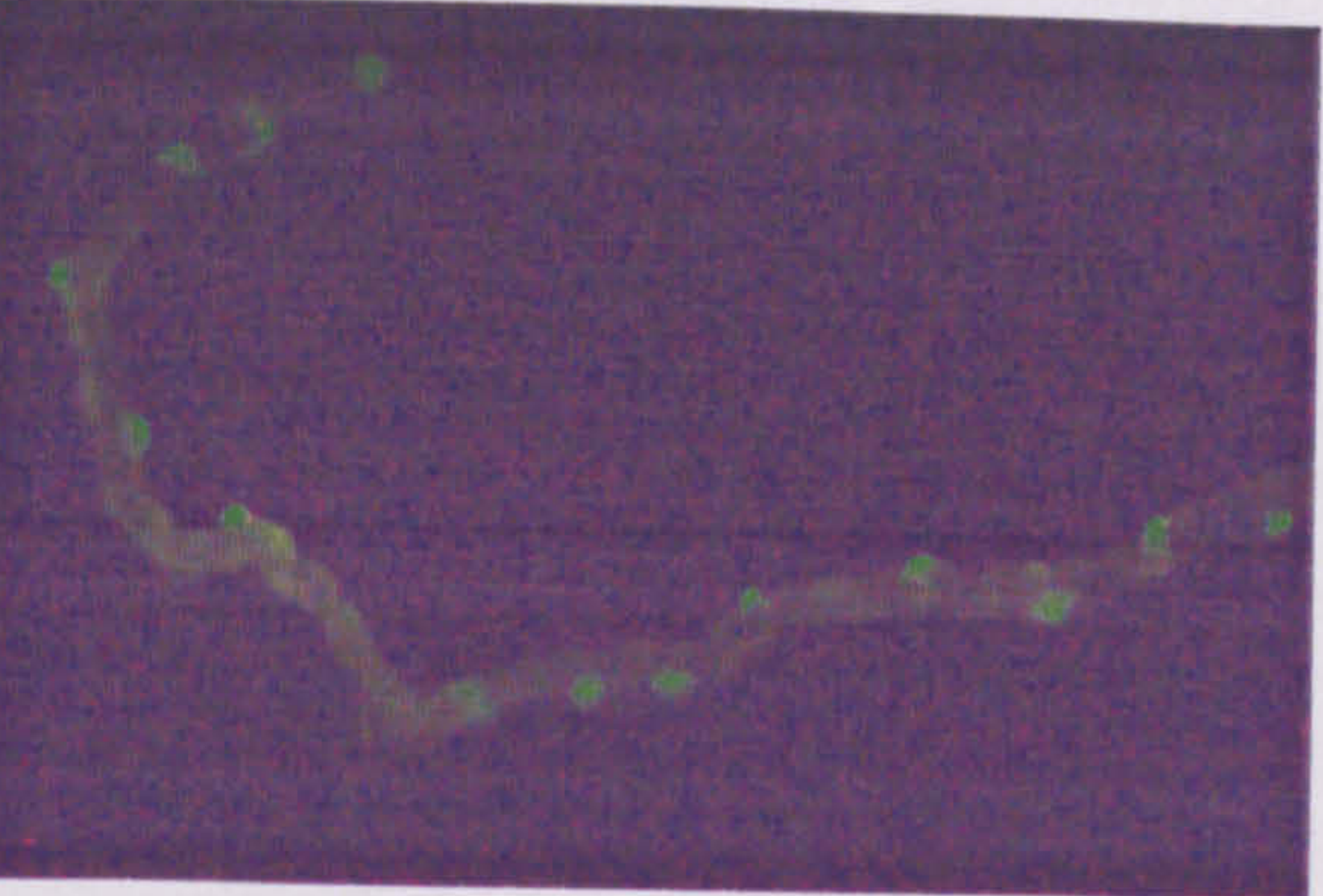
f)



g)



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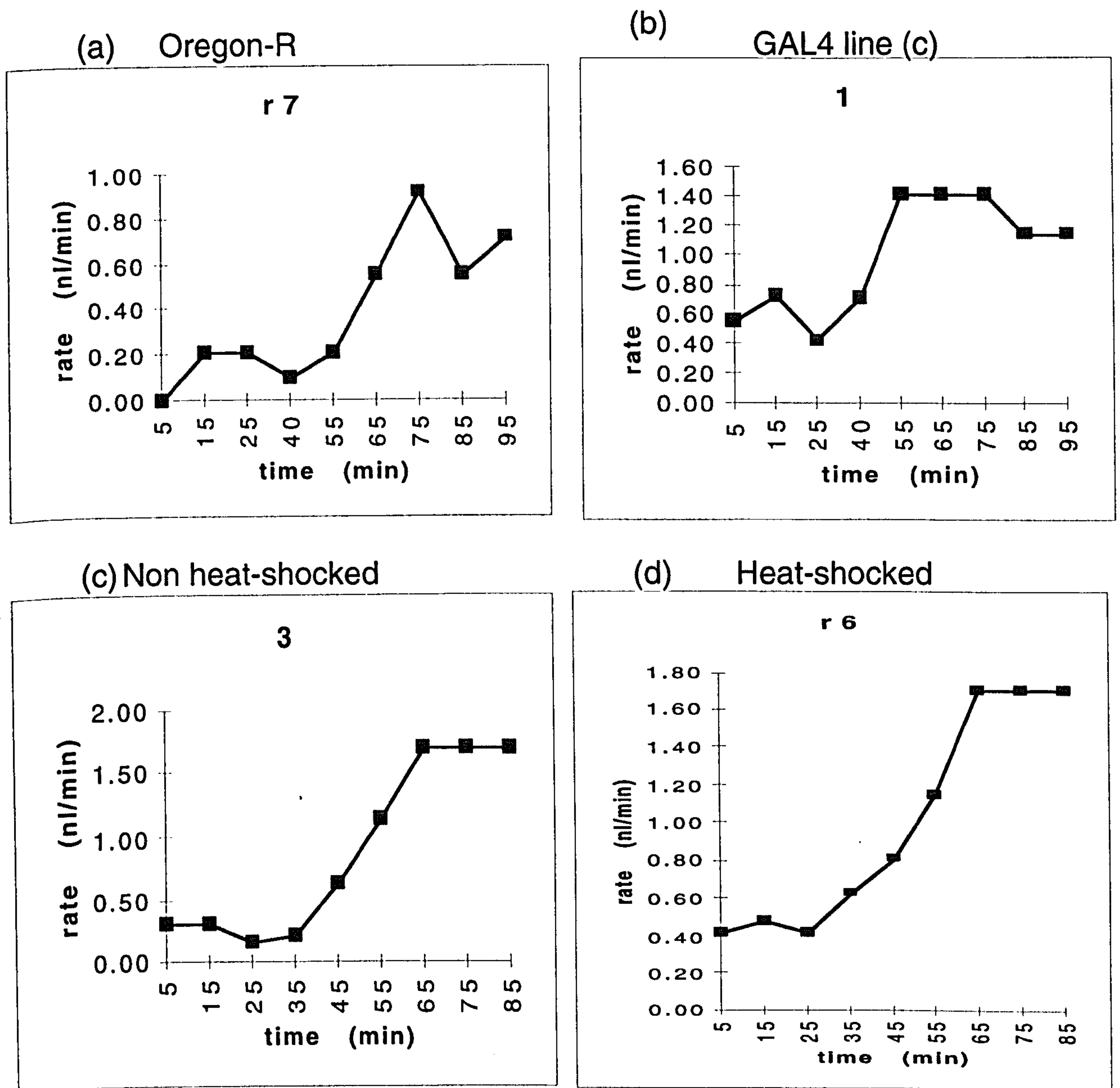


Figure 3.37. Testing ablation physiologically by tubule secretion assay
 (a) Oregon R control line. (b) Original GAL4 line as an another control (c) The non heat-shocked ones. (d) The heat-shocked flies from Line C724 expressed only in stellate cells.

3.7.4. *In situ* hybridisation to polytene chromosomes

In order to determine the chromosomal localisation of the main lines used in this study as a result of *in situ* hybridisation to the polytene chromosomes of salivary gland using biotin labelled pBluescript probes the following data were obtained. The lines with similar expression patterns were usually found to reside at distinct loci, usually on different chromosomes. As can be seen in this study, 3 stellate cell specific insertions (**Figs 3.31 a, b, c**) and at least 3 lines specific to some principal cells of Malpighian tubules were observed to have inserted at different loci. Only one expression pattern (in the lower tubule) reported by two lines appeared to result from independent insertion at the same chromosomal position. **Table 3.6** shows the rest of the lines which have interesting expression patterns used in this study.

Figure 3.32. Some examples to the chromosomal localisations using *in situ* hybridisation technique of the main lines used in this study.

a,b) The chromosomal localisation of line C724 expressed in stellate cells is 40C near the centromere of chromosome 2L.

c) Line C710, though also expressed in stellate cells has a distinct chromosomal location at 66D on chromosome 3R.

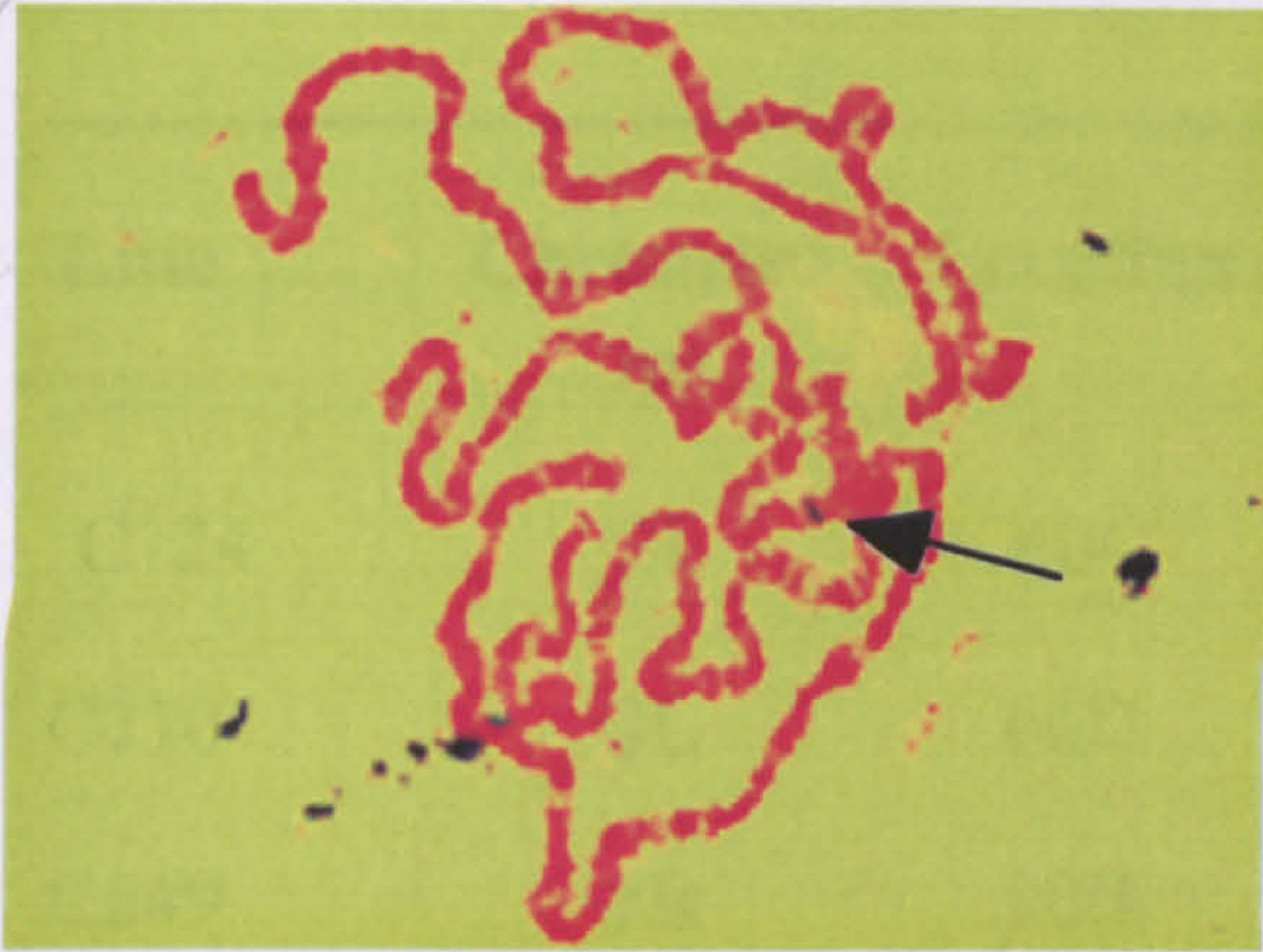
d) A higher magnification of the chromosomal localisation of line C710.

e) Line C324's chromosomal localisation (23B on chromosome 2L) which marks a subset of principal cells.

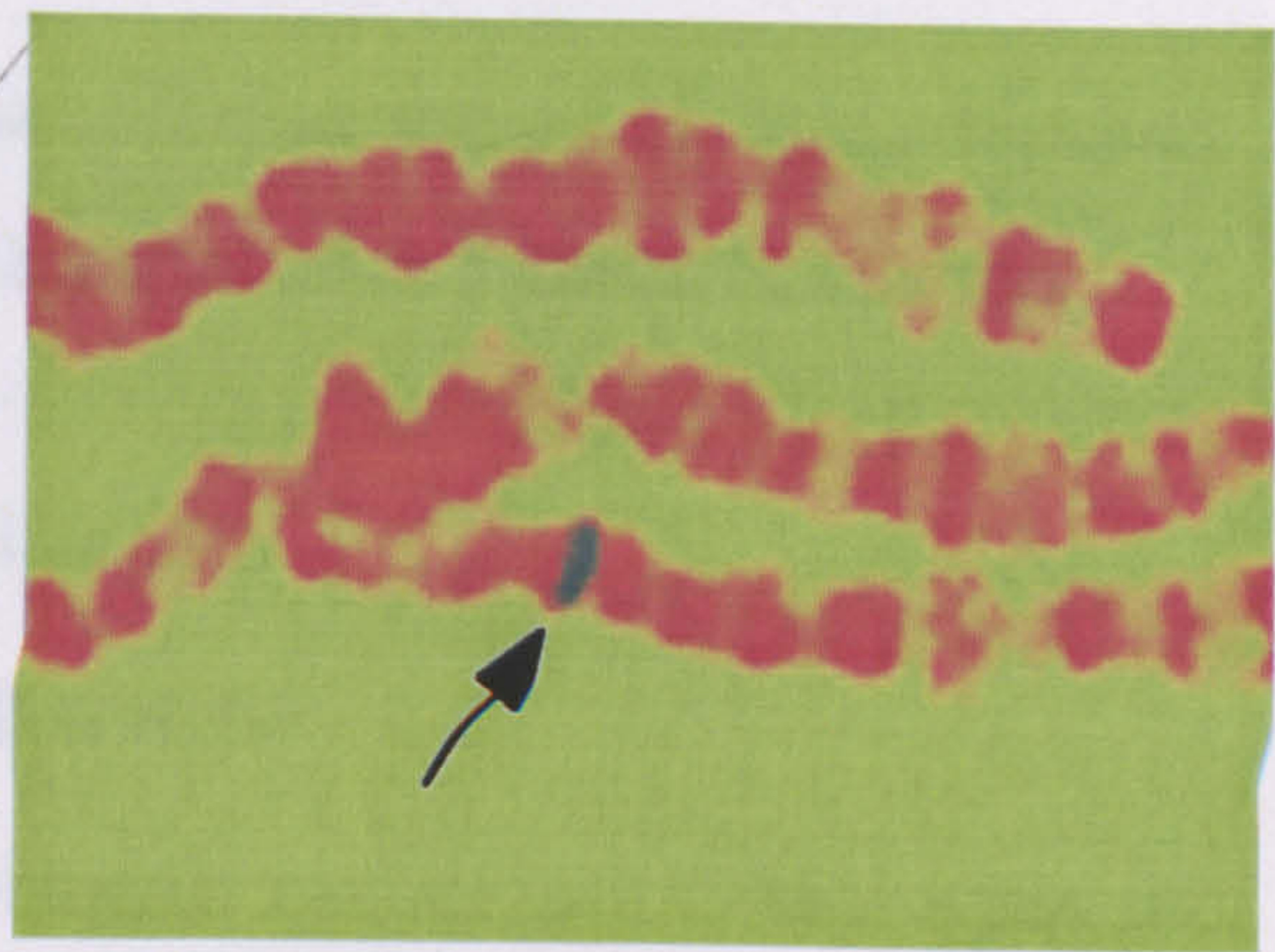
f) A higher magnification of the chromosomal localisation of line C324.

Table 3.6. Chromosomal localization of the 250k probe used in this study.

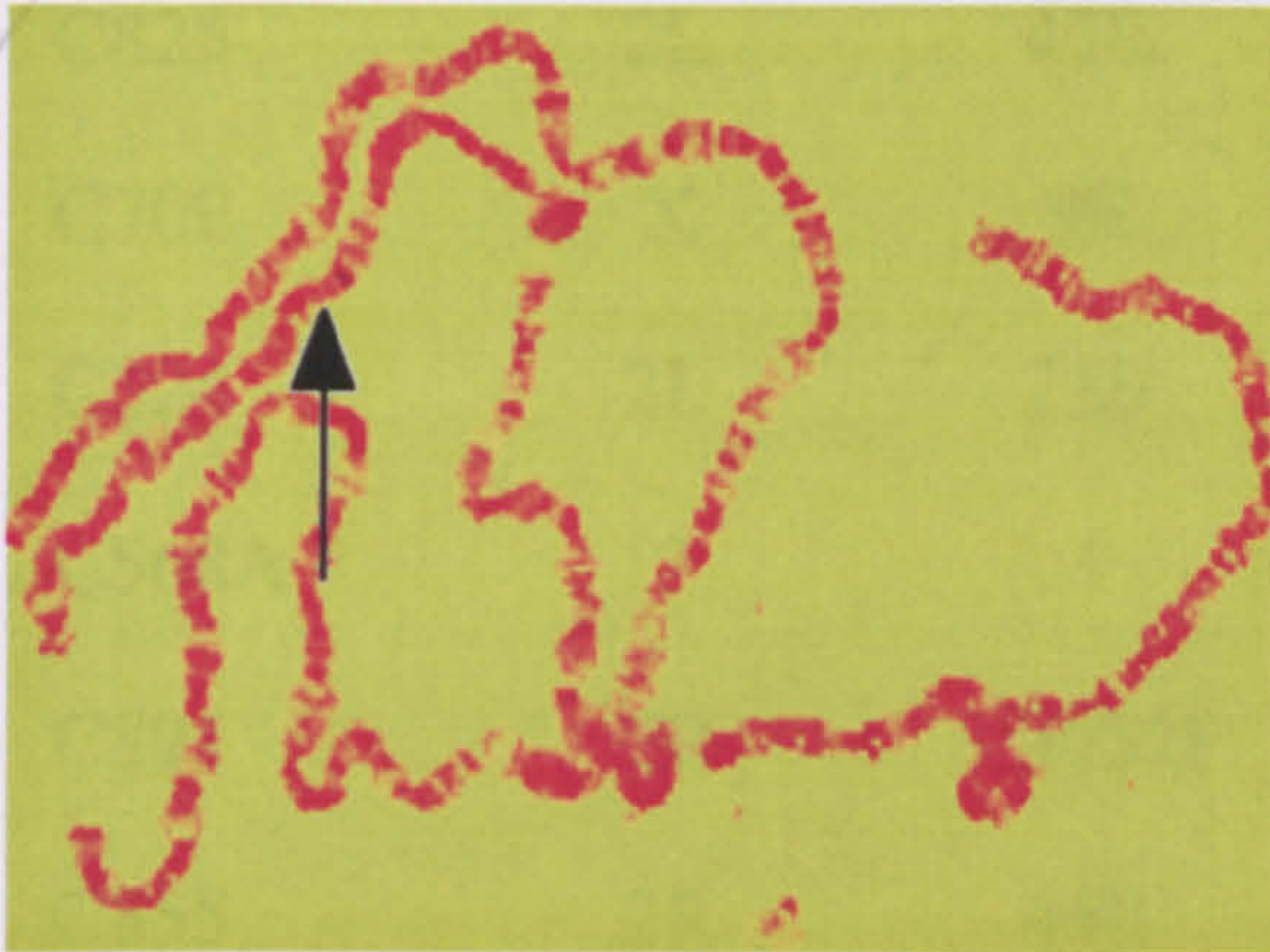
a)



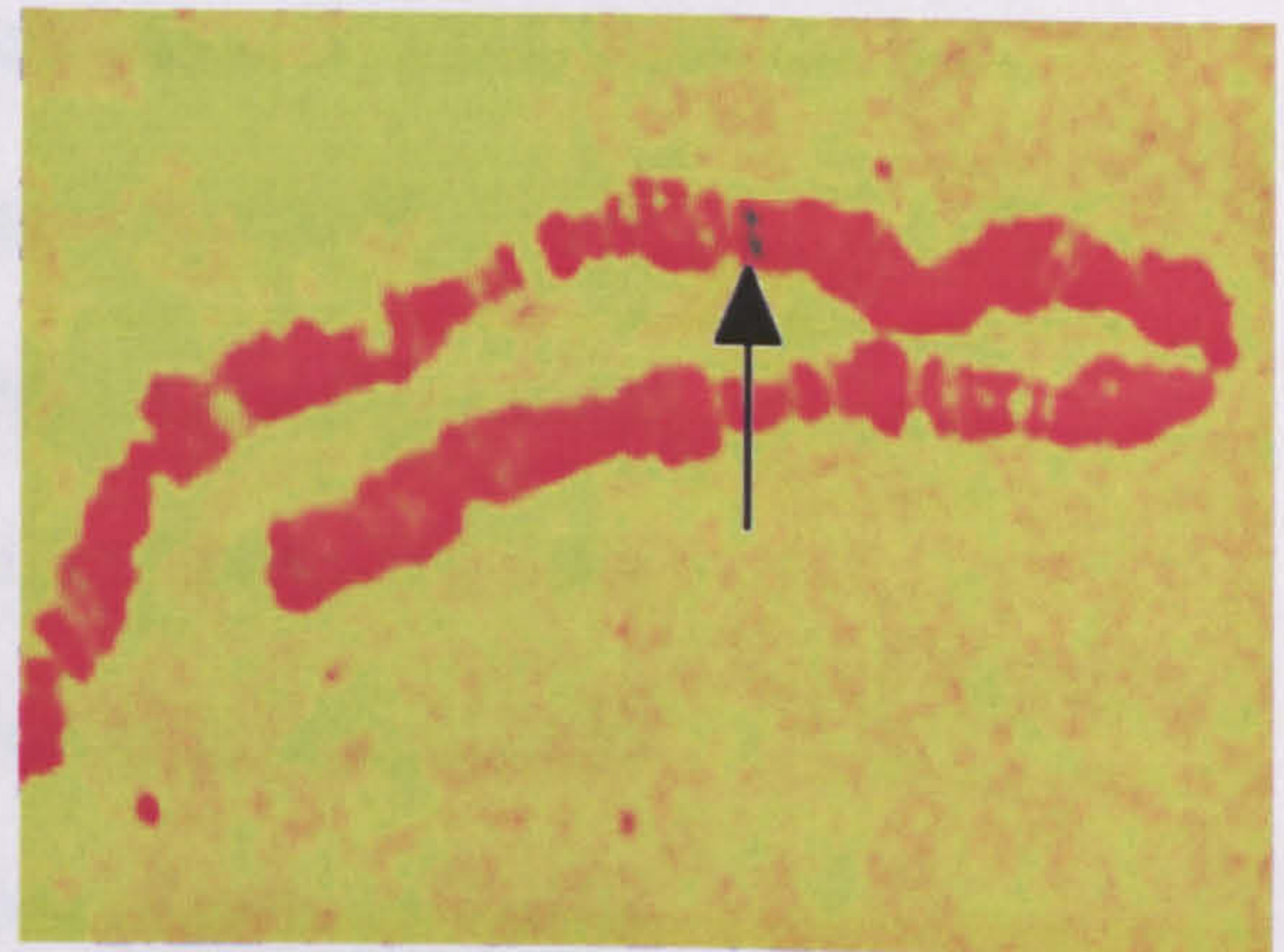
b)



c)



d)



e)



f)



Table 3.6. Chromosomal localisations of the main lines used in this study.

Line	Chromosome	Location	<i>LacZ</i> expression pattern in tubules
C724	2L	40C	Stellate cells
C710	3L	66D	Stellate cells
C649	3R	90B	Bar-shaped stellate cell homologues of initial, transitional segments
C825	2L	45E	Initial segment, main segment, gut
C709	X	9E	All principal cells
C324	2L	23B	Subset of principal cells
C568A	?	?	Subset of principal cells
C801	2R	55F/57F	Subset of principal cells
C855	2R	46E	Subset of principal cells
C374	2R	55C	Subset of principal cells
C507	3R	100B	Lower tubule and ureter
C232	3R	100B	Lower tubule and ureter
C803	3L	79F	Tiny cells
C42	?	?	Principal cells except initial and transitional area, bar-shaped cells

3.8. Preliminary molecular characterization of some of the candidate genes specifically expressed in Malpighian tubules

As a first step to the molecular characterisation of some of enhancer trap lines specifically expressed in Malpighian tubules, either subtypes or subregions, 8 of them were chosen for plasmid rescue. Different sizes of genomic flanking sequences were obtained using mainly 2 restriction enzymes (Pst I in **Fig 3.33** and, Sst I in **Figure 3.34**) as shown altogether in **Table 3.7** in the following section with the original plasmid, pGawB on page 116.

Because of limited time, a single line (C724) was selected for more detailed study. A more complete restriction map was assembled (**Figure 3.35**) and used as a basis for a subcloning strategy, designed to yield fragments suitable for sequencing (**Figure 3.36**).

Using a combination of different restriction enzymes for line C724 expressed specifically in stellate cells in all stages (from late embryo to adult), restriction maps of rescued plasmids were obtained (**Figure 3.35**).

Then the same fragments were used for subcloning in order to sequence. The restriction mapping and subcloned fragments are shown in **Figure 3.36** with their explanations.

The authenticity of subcloned fragments was established by Southern blot hybridisation with a probe prepared from the original rescued plasmid (**Figs 3.37 a-d**).

Southern blotting was also used for screening of the genomic P1 clones mapped to this genomic region (**Figs 3.37 e-f**).

Figure 3.33. Some of the single and double digests of rescued plasmids with Pst I.

Note that in this figure for the enzyme, Sac II as its another name , "Sst II", is also used. The standard (1 kb) ladder (from Gibco BRL) was used sometimes at the beginning (as in a,e and g), and sometimes in the end (as in c). The lanes show as follows:

Lane 1: Undigested rescued plasmid DNA.

Lane 2: Rescued plasmid DNA single digested with Pst I.

Lane 3: Rescued plasmid DNA single digested with Sst II (Sac II).

Lane 4: Rescued plasmid DNA double digested with Pst I and Sst II (Sac II).

a) Line c724 rescued with Pst I

b) Schematic expression of the rescued plasmid DNA together the genomic flanking sequence.

c) Line c374 rescued with Pst I.

d) Schematic expression of the rescued plasmid DNA with its genomic insert.

e) Line c801 rescued with Pst I.

f) Schematic representation of the rescued plasmid DNA along with its genomic flanking DNA.

g) Line c803 rescued with Pst I.

h) Schematic expression of the rescued plasmid DNA with its genomic insert DNA.

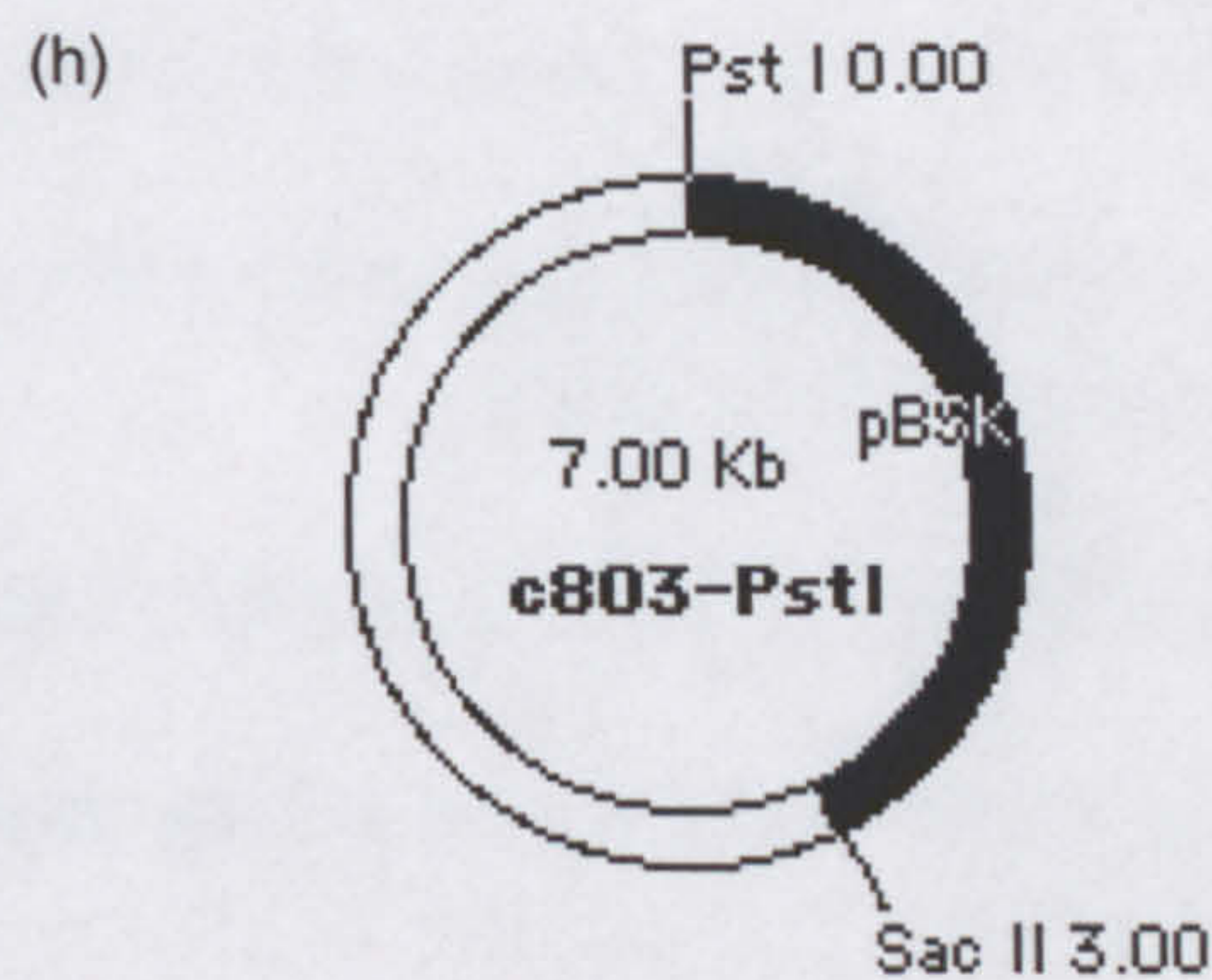
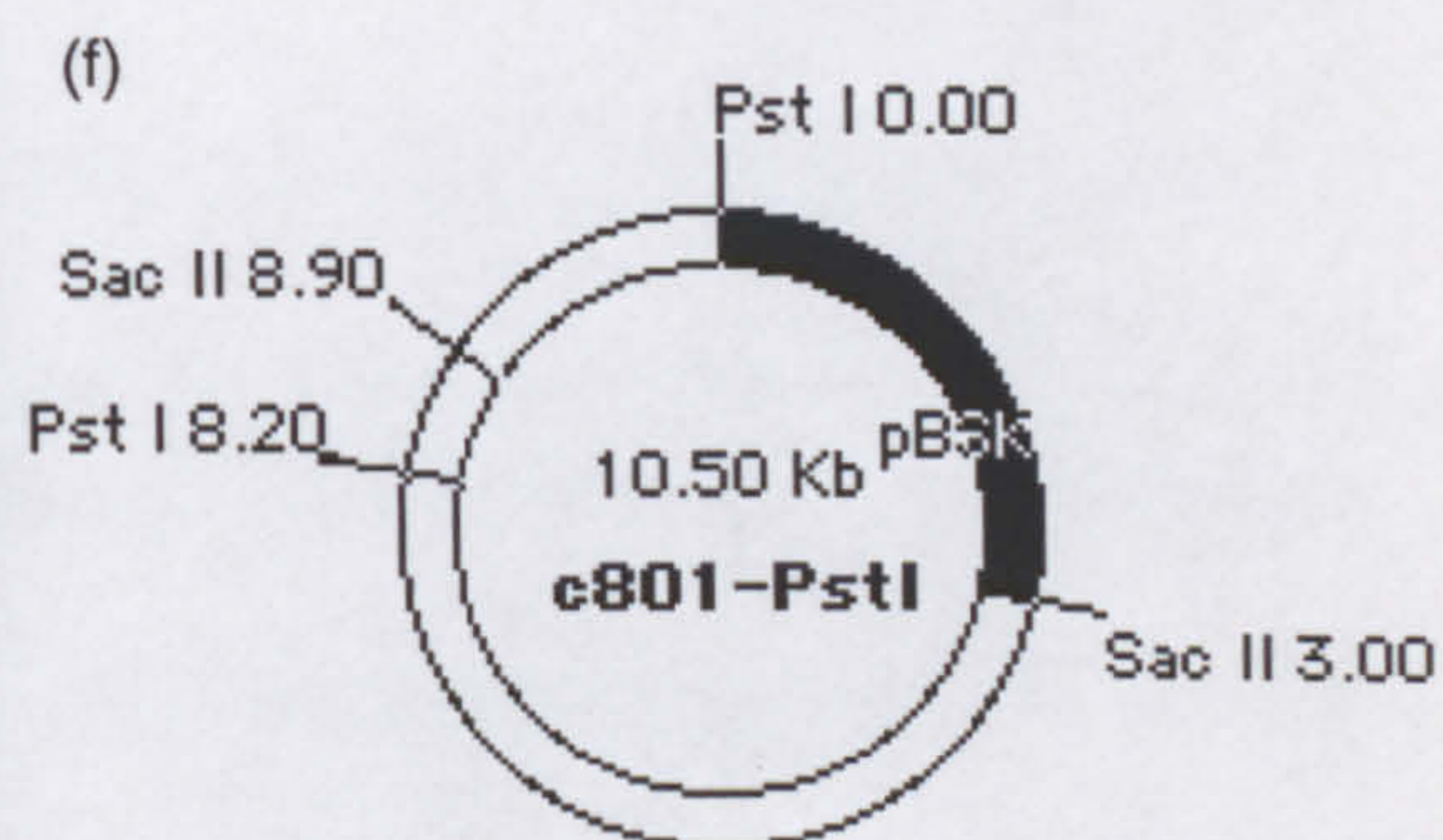
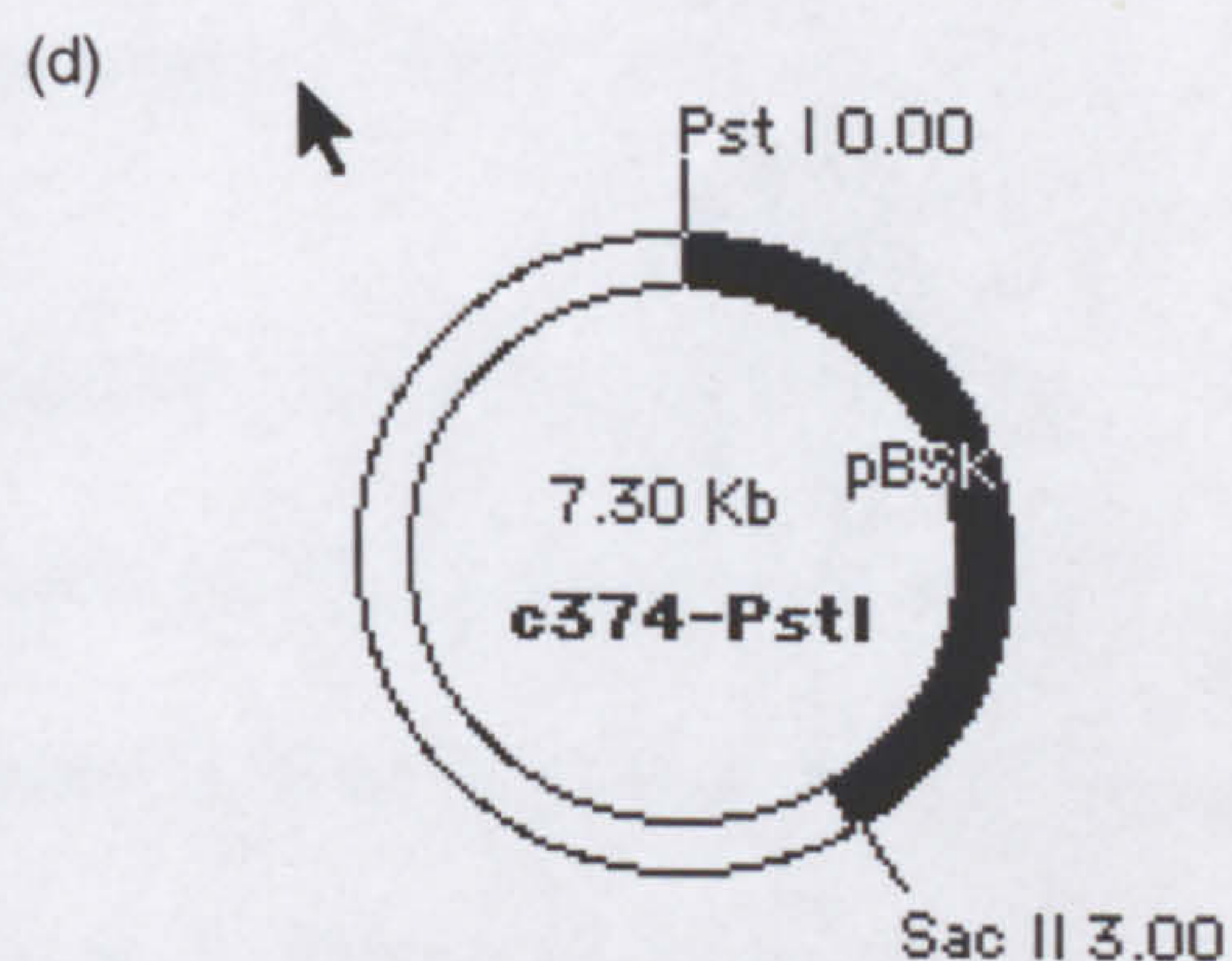
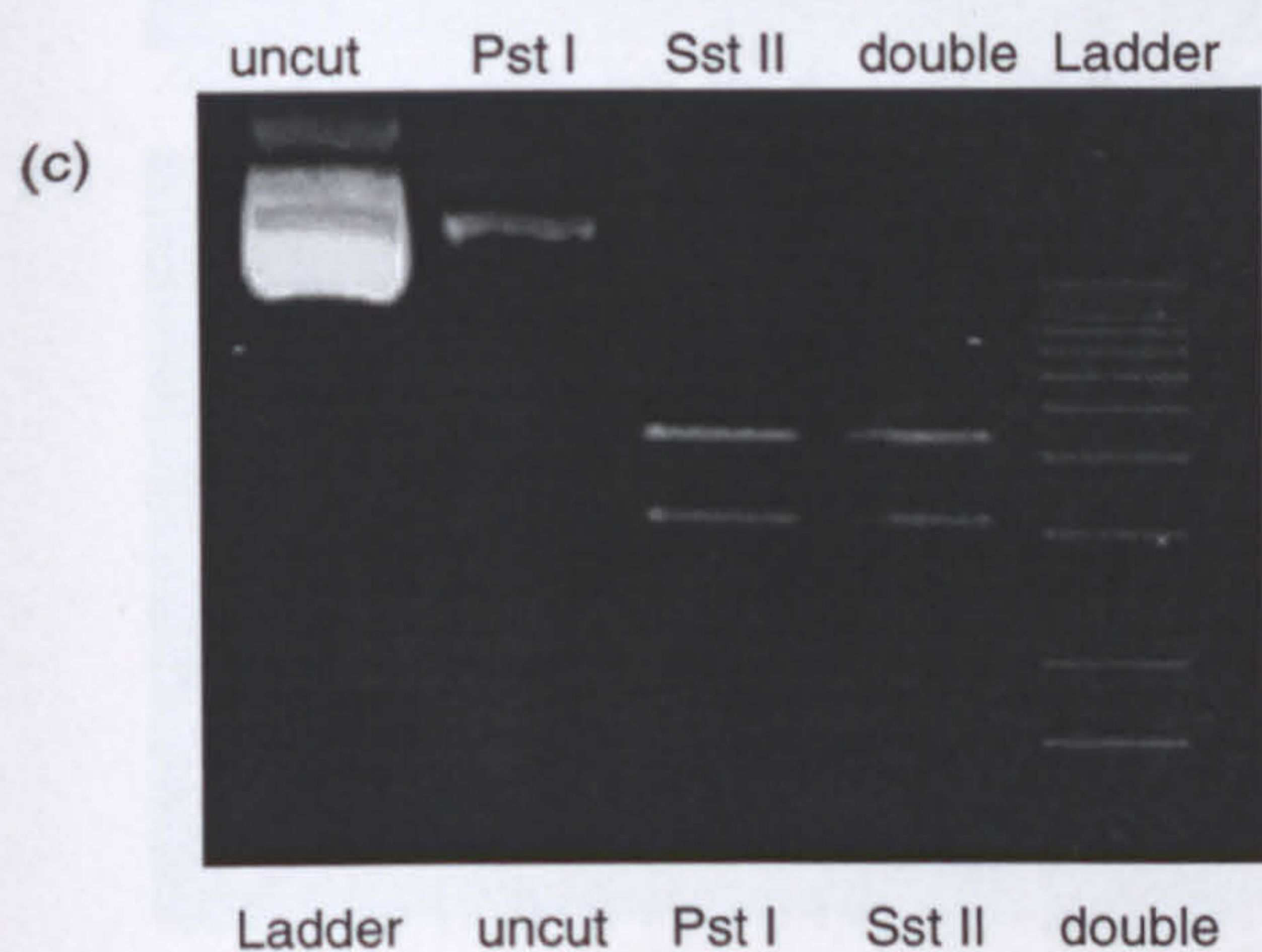
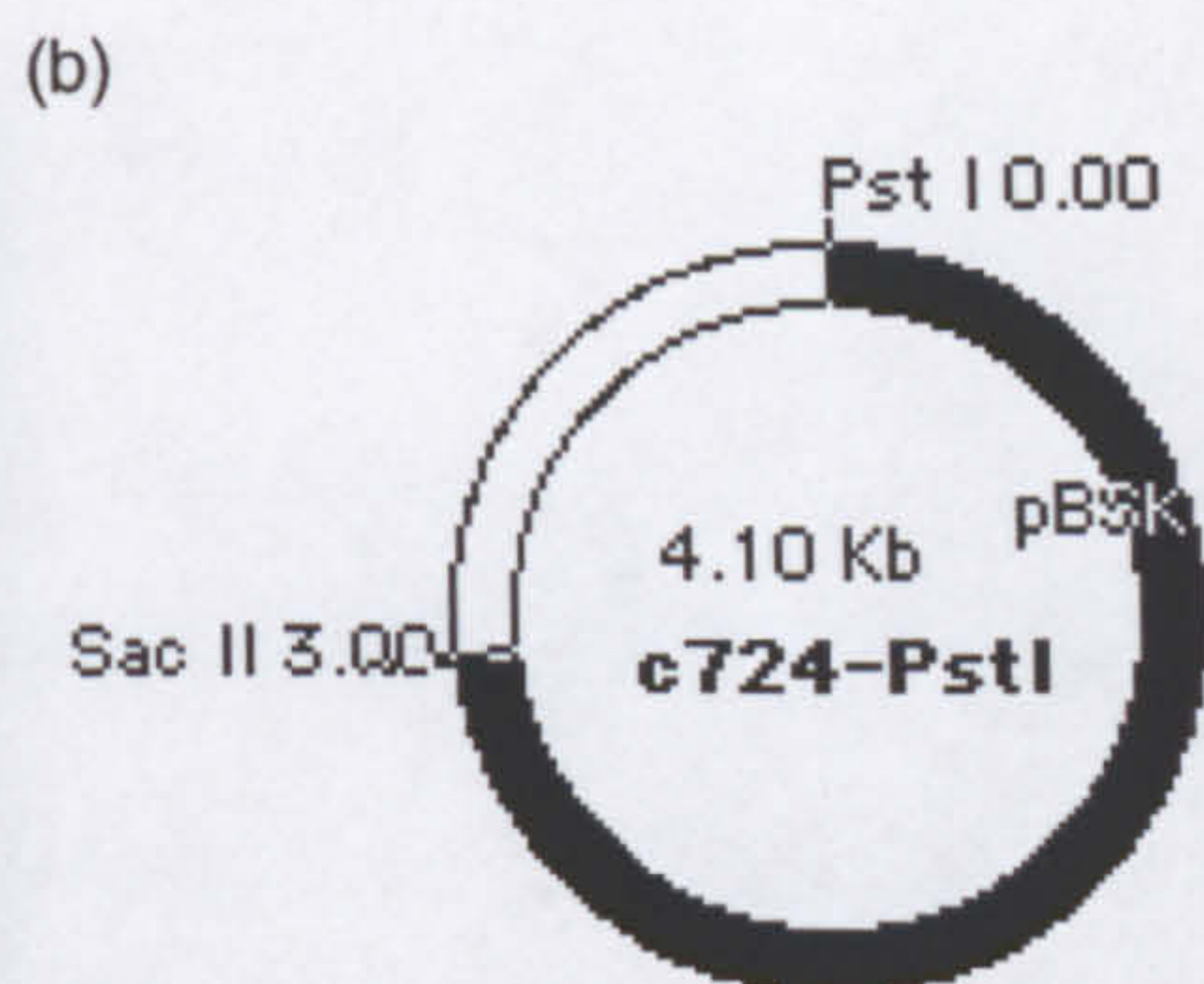


Figure 3.34. Some of the rescued plasmids with Sac I (Sst I)

Rescued plasmids with Sac I (note that Sst I is also used for the same enzyme in this figure) were represented in the following way.

The standard (1 kb) ladder (from Gibco BRL) was used sometimes at the beginning (as in a,c and e), and sometimes in the end (as in g). The lanes show as follows:

Lane 1: Undigested rescued plasmid DNA.

Lane 2: Rescued plasmid DNA single digested with Sac I (Sst I).

Lane 3: Rescued plasmid DNA single digested with Sac II (Sst II).

Lane 4: Rescued plasmid DNA double digested with Sac I (Sst I) and Sac I (Sst II).

a) Line c724 rescued with Sac I (Sst I).

b) Schematic representation of the rescued plasmid DNA together the genomic flanking sequence by digestion of suitable enzymes.

c) Line c710 rescued with Sac I (Sst I).

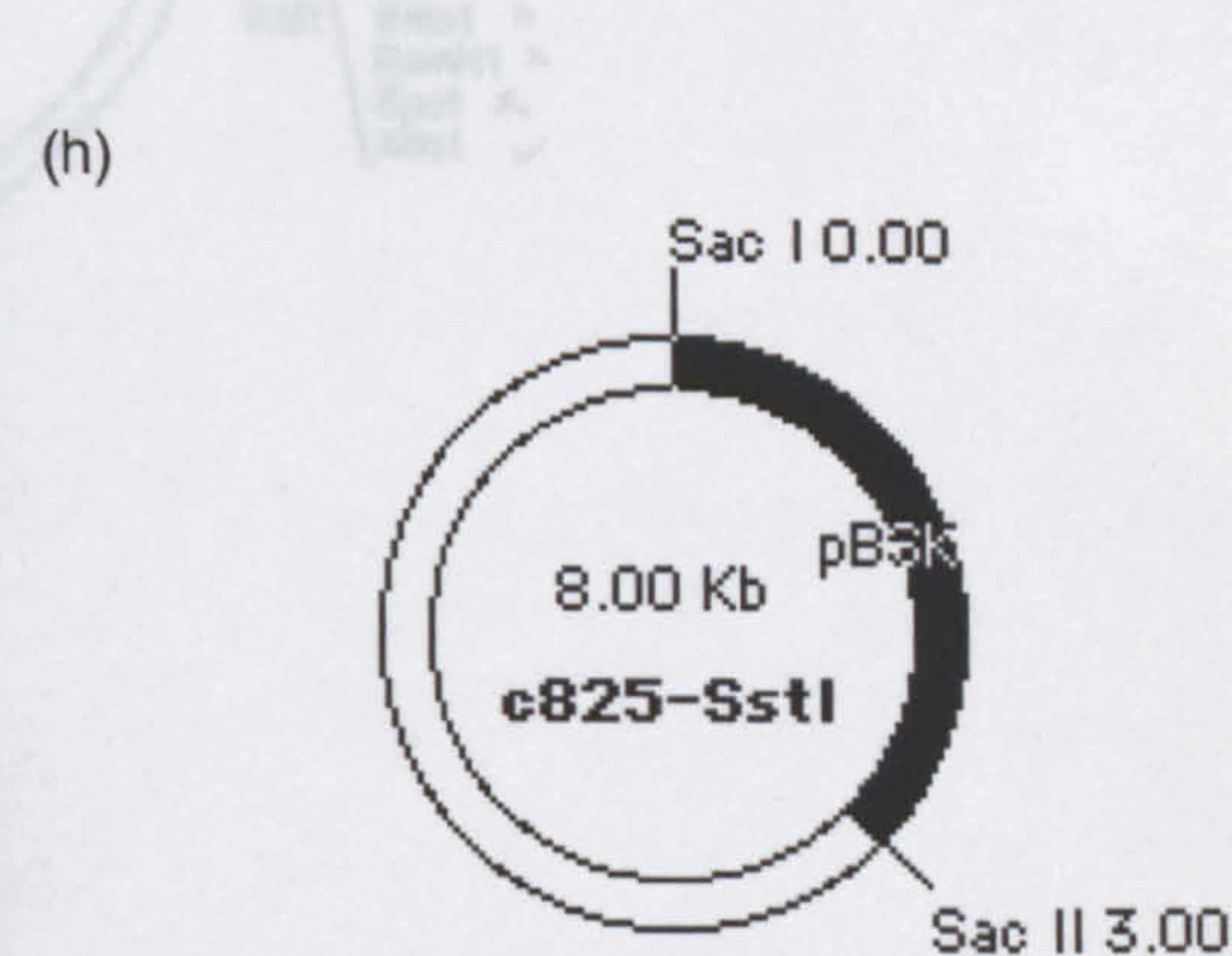
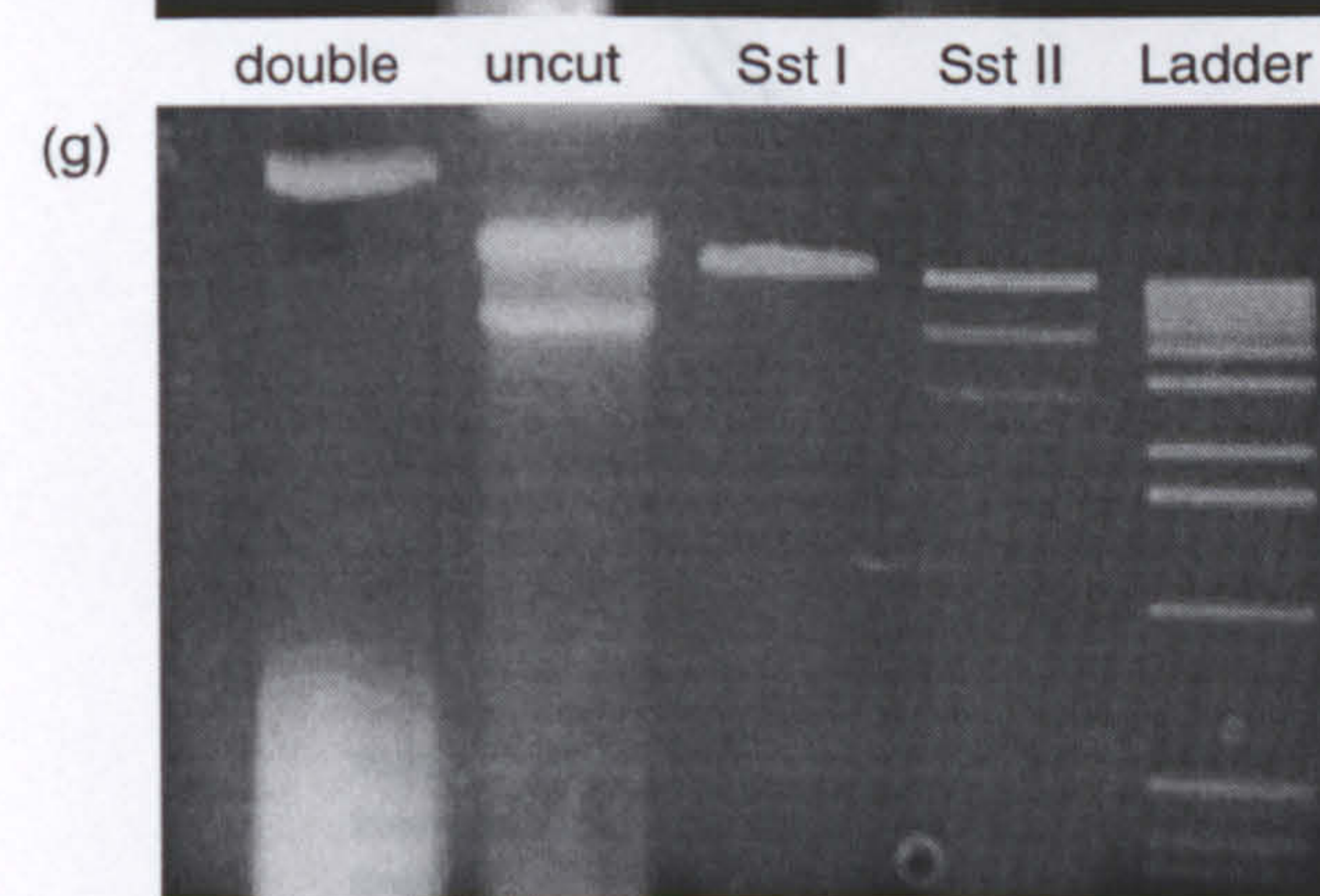
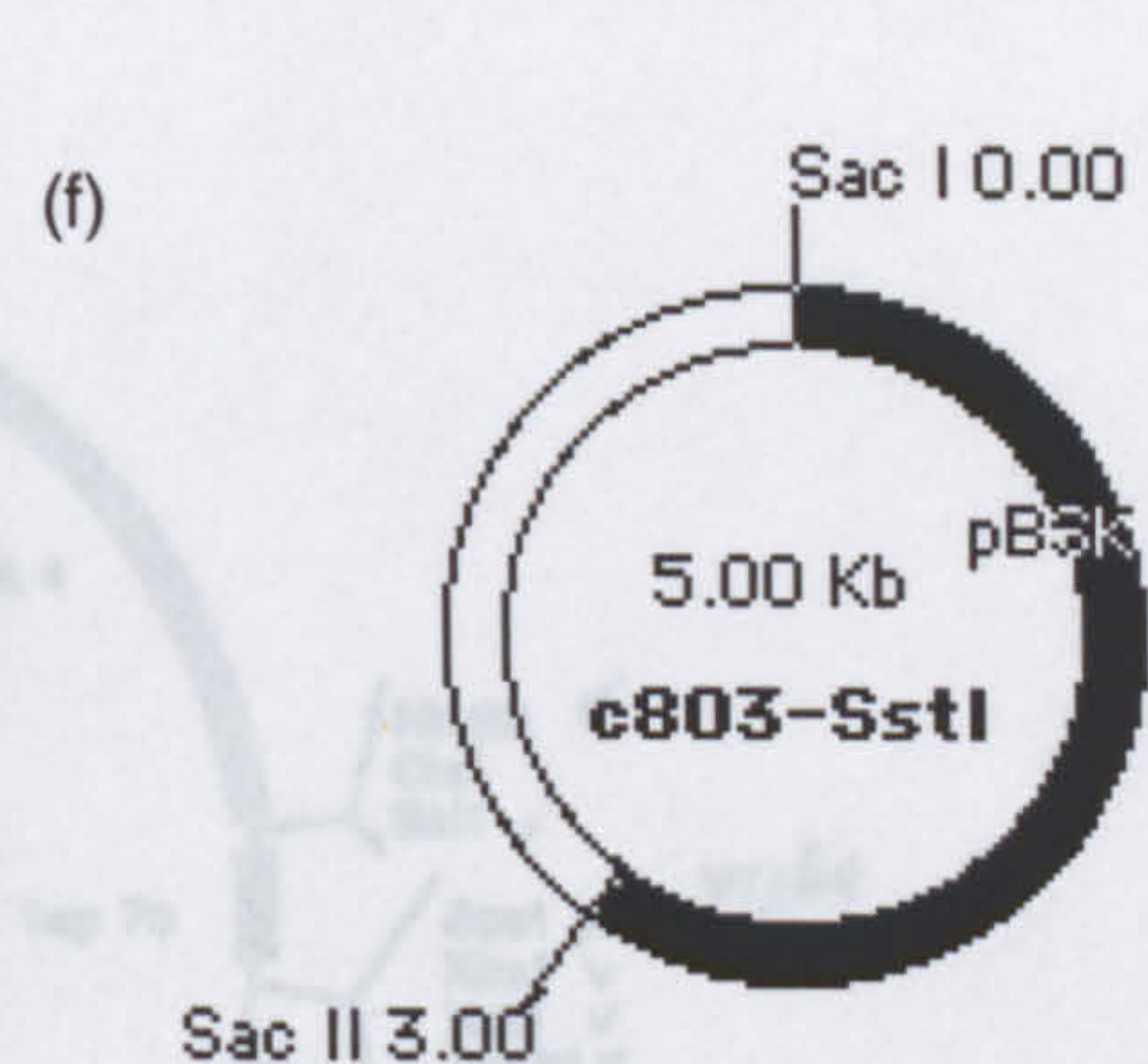
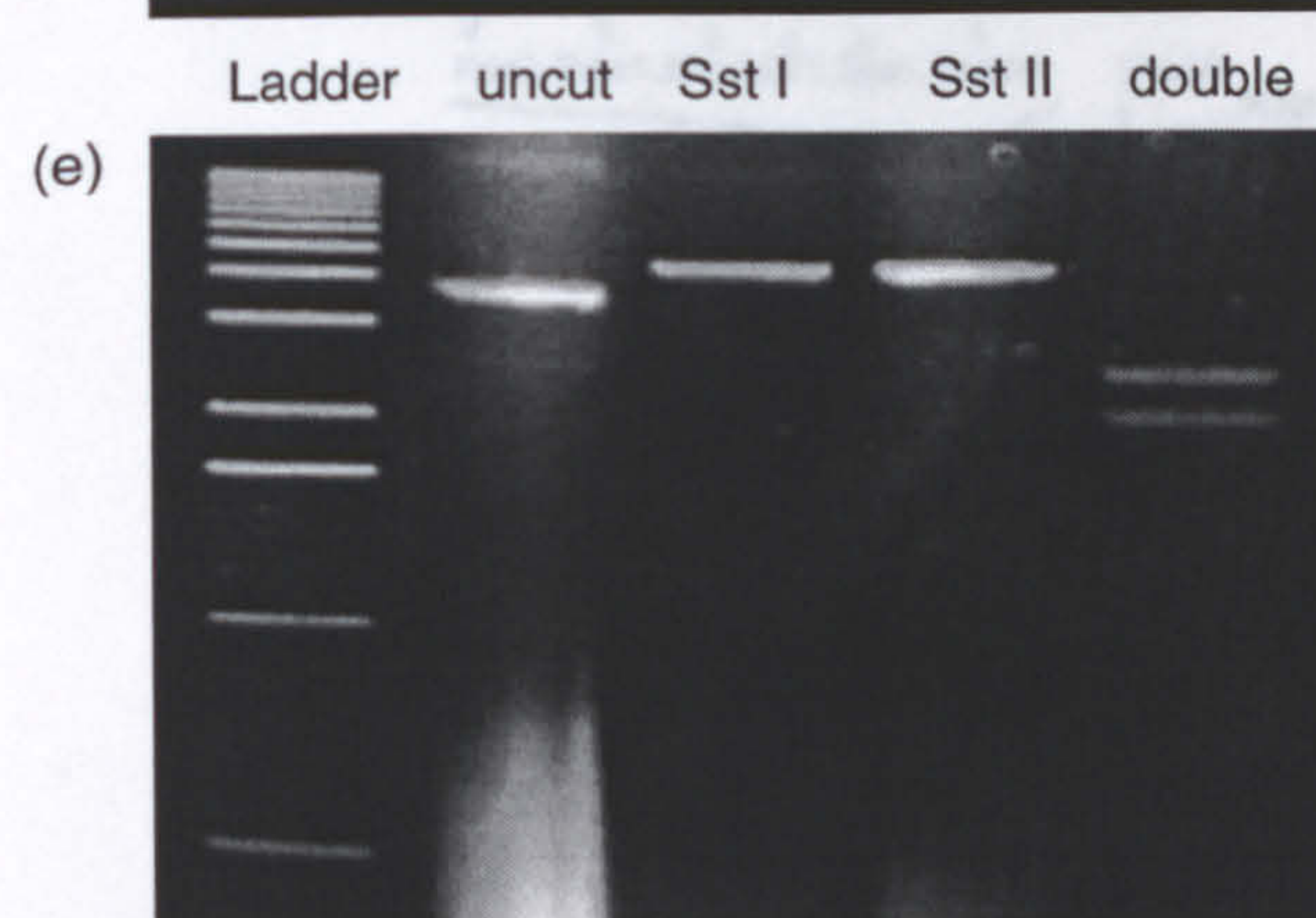
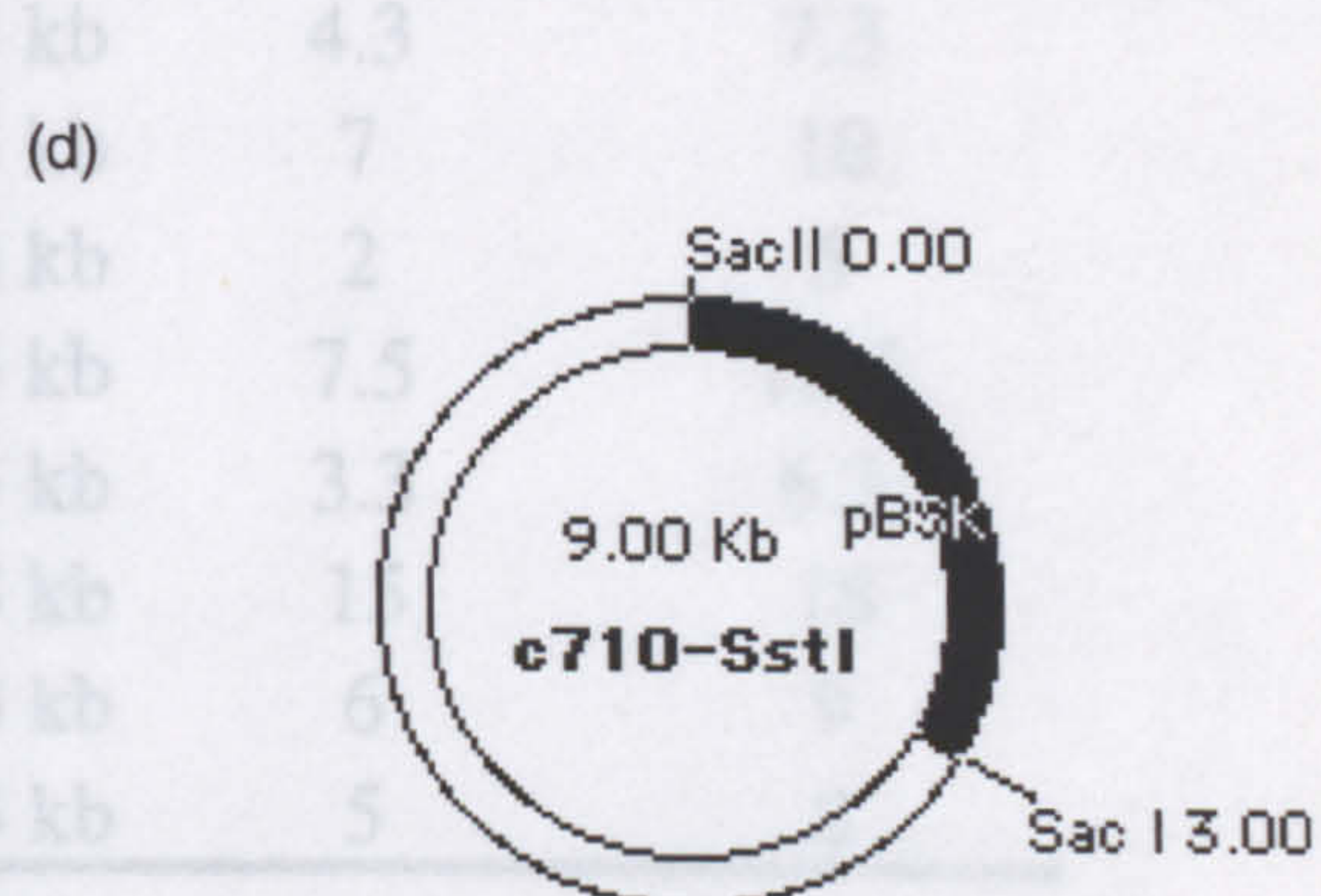
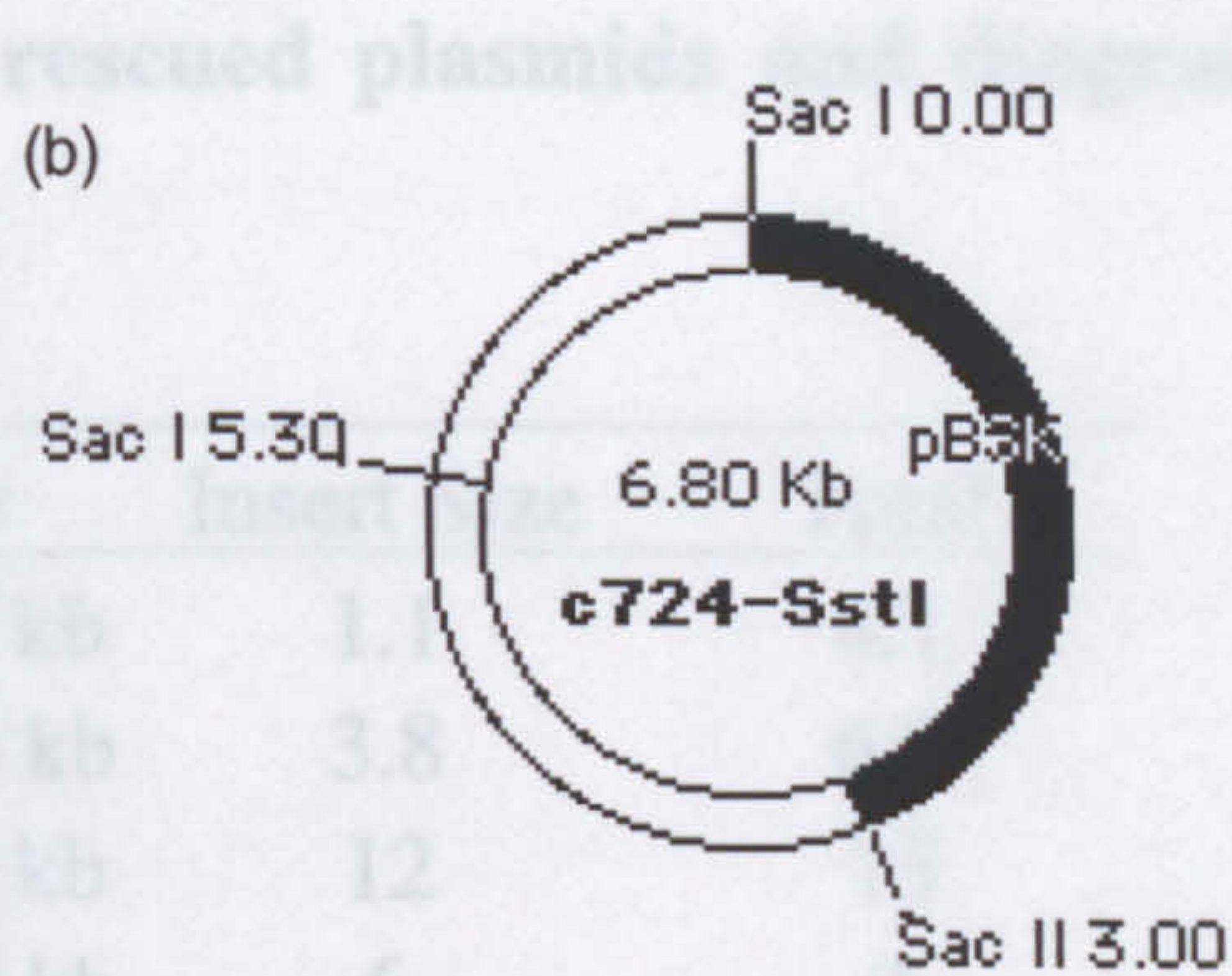
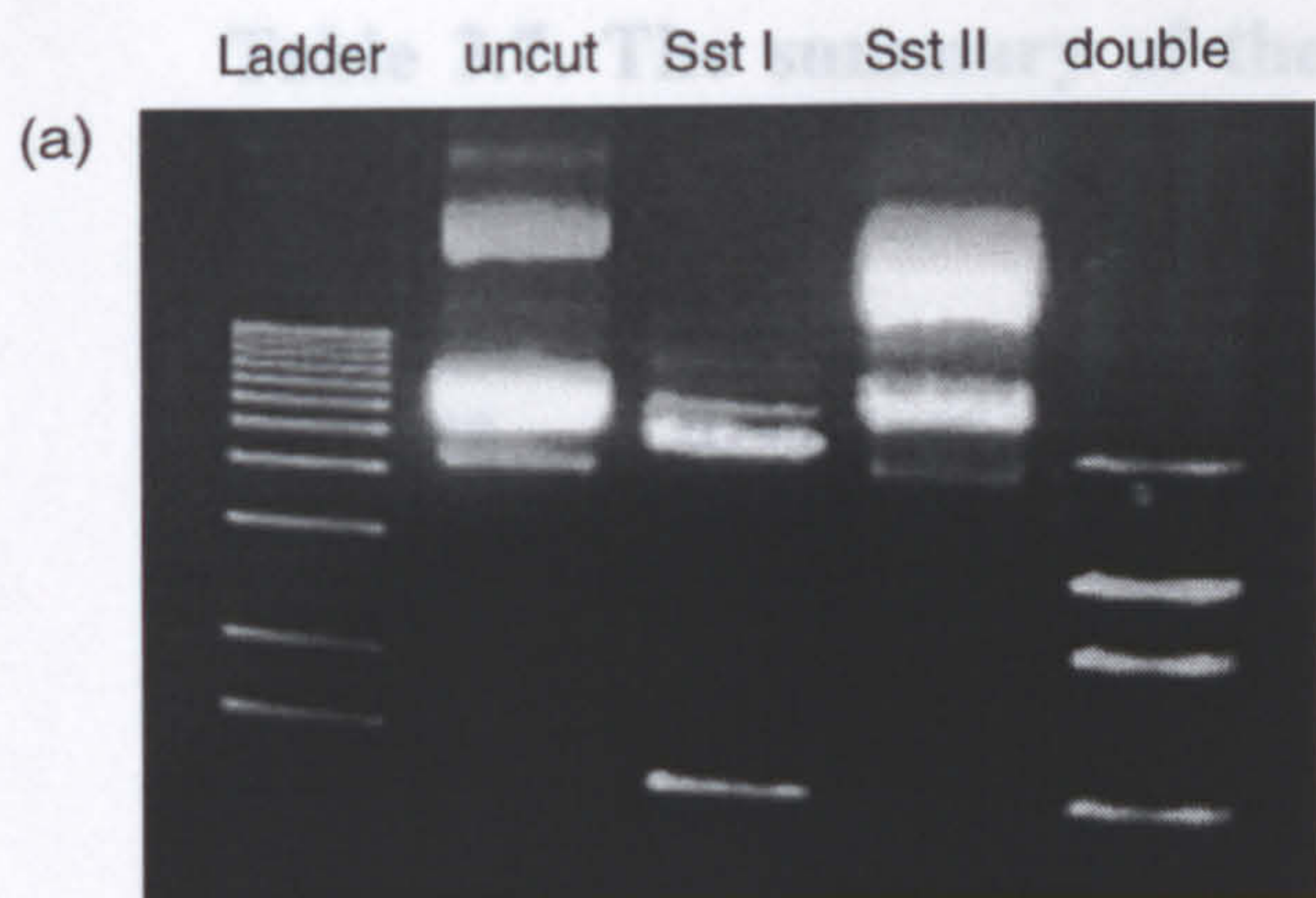
d) Schematic expression of the rescued plasmid DNA with its genomic insert.

e) Line c803 rescued with Sac I (Sst I).

f) Schematic representation of the rescued plasmid DNA along with its genomic flanking DNA.

g) Line c825 rescued with Sac I (Sst I).

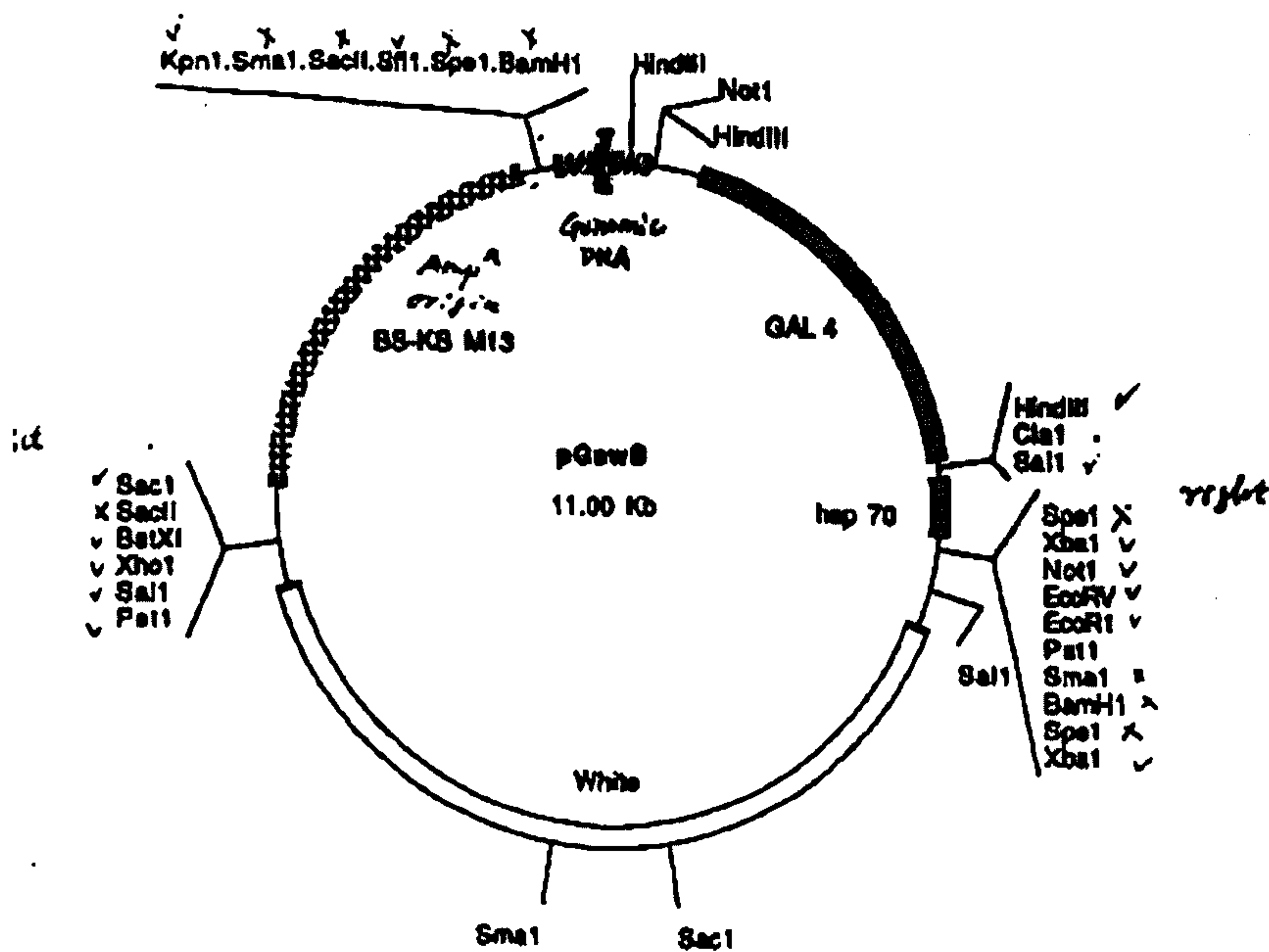
h) Schematic expression of the rescued plasmid DNA with its genomic insert DNA.



Construction of c724-SstI
Construction of c710-SstI

Table 3.7. The summary of the rescued plasmids and diagram of PGaw B

Line	R. Enzyme	Vector	Insert Size	Total
C724	Pst I	pBSK/ 3 kb	1.1	4.1
C724	Sac I	pBSK / 3 kb	3.8	6.8
C710	Pst I	pBSK /3 kb	12	15
C710	Sac I	pBSK / 3 kb	6	9
C374	Pst I	pBSK / 3 kb	4.3	7.3
C803	Pst I	pBSK / 3 kb	7	10
C803	Sac I	pBSK / 3 kb	2	5
C801	Pst I	pBSK / 3 kb	7.5	10.5
C801	Sac I	pBSK / 3 kb	3.3	6.3
155Y	Sac I	pBSK / 3 kb	15	18
C649	Sac I	pBSK / 3 kb	6	9
C825	Sac I	pBSK / 3 kb	5	8



Plasmid name: pGawB
Plasmid size: 11.00 kb
Constructed by: A.H.Brand and N.Perrimon
Construction date: 7
Comments/References: Development 118, 401-415 (1993)

Figure 3.35. Restriction mapping of line c724.

Rescued plasmids with Sac I were represented in the following way.

The standard (1 kb) ladder (from Gibco BRL) was used sometimes at the beginning (as in a,c and e), and sometimes in the end (as in g). The lanes show as follows:

Lane 1: Undigested rescued plasmid DNA.

Lane 2: Rescued plasmid DNA single digested with Sac I (Sst I).

Lane 3: Rescued plasmid DNA single digested with Sac II (Sst II).

Lane 4: Rescued plasmid DNA double digested with Sac I and Sac II.

a) Restriction digests of Sac I (lane 1,3,6) and double digests of Sst I and Sac II (lane 2,4,7,8) with standard 1 kb DNA ladder (BRL).

b) Schematic representation of restriction digests with Sac I and Sac II.

c) Restriction digests of EcoR V, Sac I, Sst II and Xba I (single digest with Xba I in lane 1, triple digest with Sst I, Sst II and Xba I in lane 2, single digest with EcoRV in lane 3, triple digest with Sst I, Sst II and EcoR V in lane 4, with standard 1 kb DNA ladder (BRL) in lane 5).

d) Schematic representation of restriction digests with Sst I, Sst II and EcoR V.

e) Restriction digests of Hind III, Sst I, Sst II(single digest with Hind III in lane 1, double digests of Hind III and Sst I in lane 2, double digests of Hind III and Sst II in lane 3, triple digest with Hind III, Sst I and Sst II in lane 4, standard 1 kb DNA ladder (BRL) in lane 5).

f) Schematic representation of restriction digests with Sst I and Sst II.

g) Restriction digests with Pst I, Hind III, Sac I and Sac II (standard 1 kb DNA ladder (BRL) in lane 1, single digest with Pst I in lane 2, single digest with Hind III in lane 3, double digests with Sac I and Sac II in lane 4, digests with 4 enzymes, Pst I, Hind III, Sac I and Sac II, in lane 5).

h) Schematic representation of restriction digests with Sst I , Sst II, Hind III, Pst I.

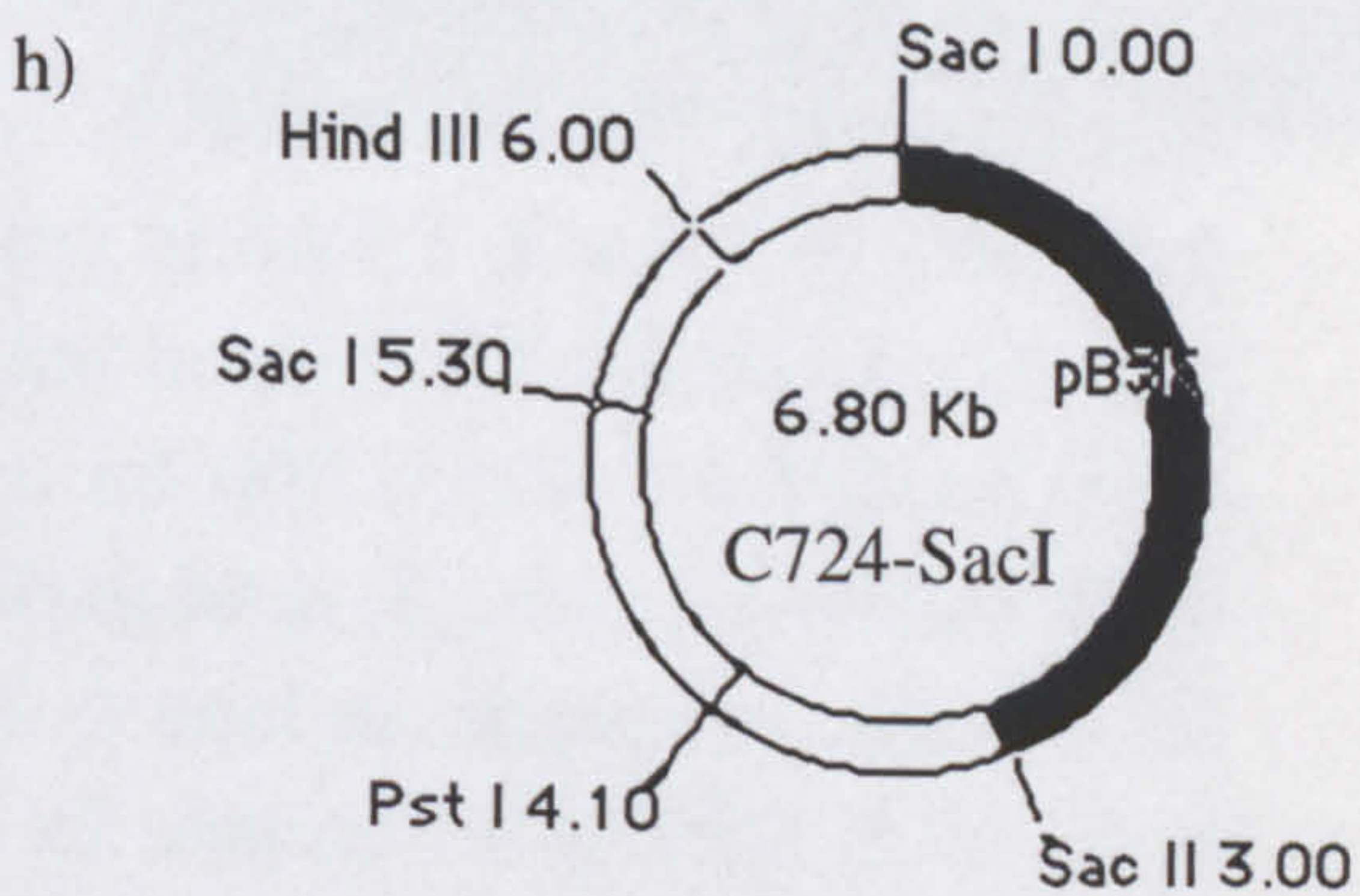
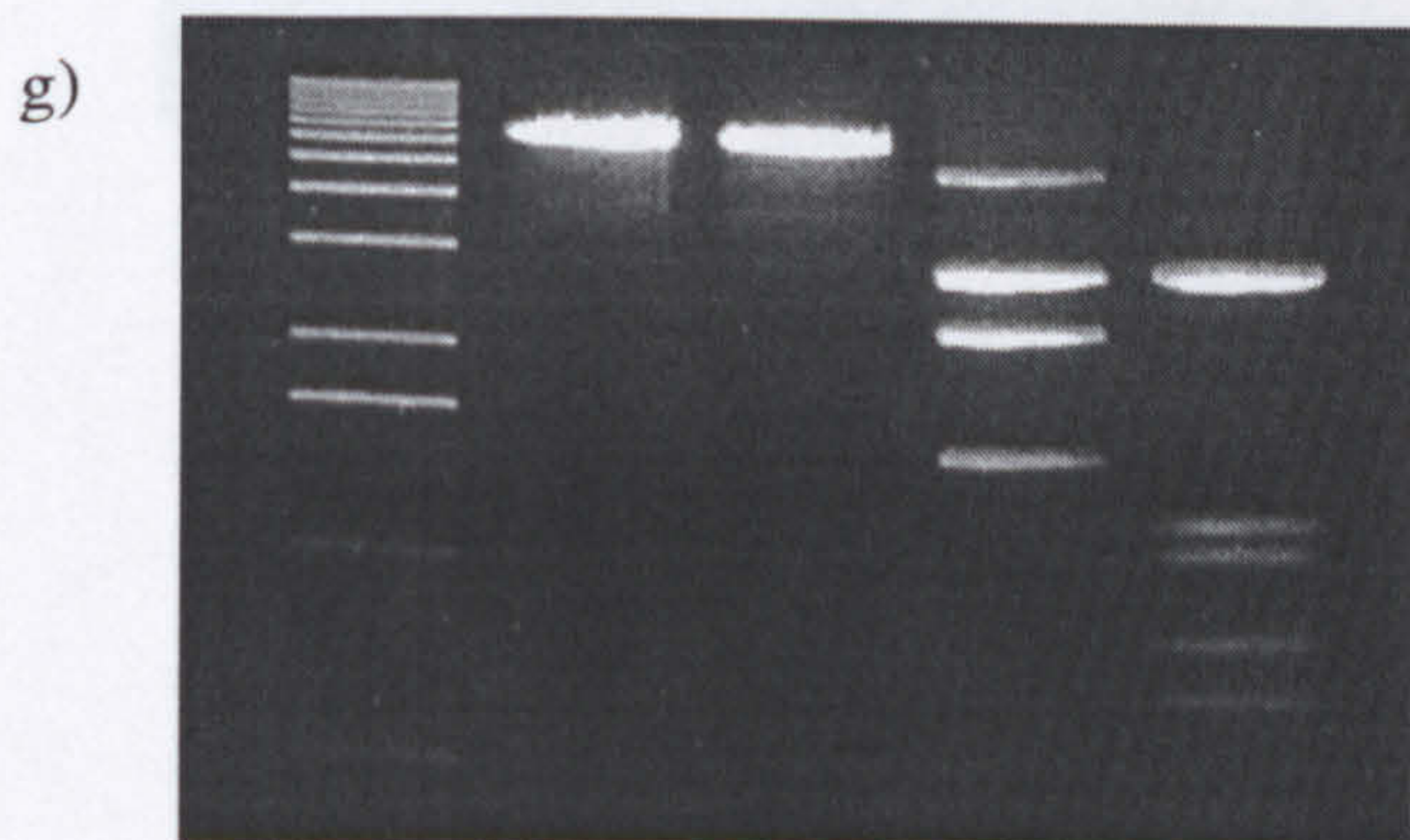
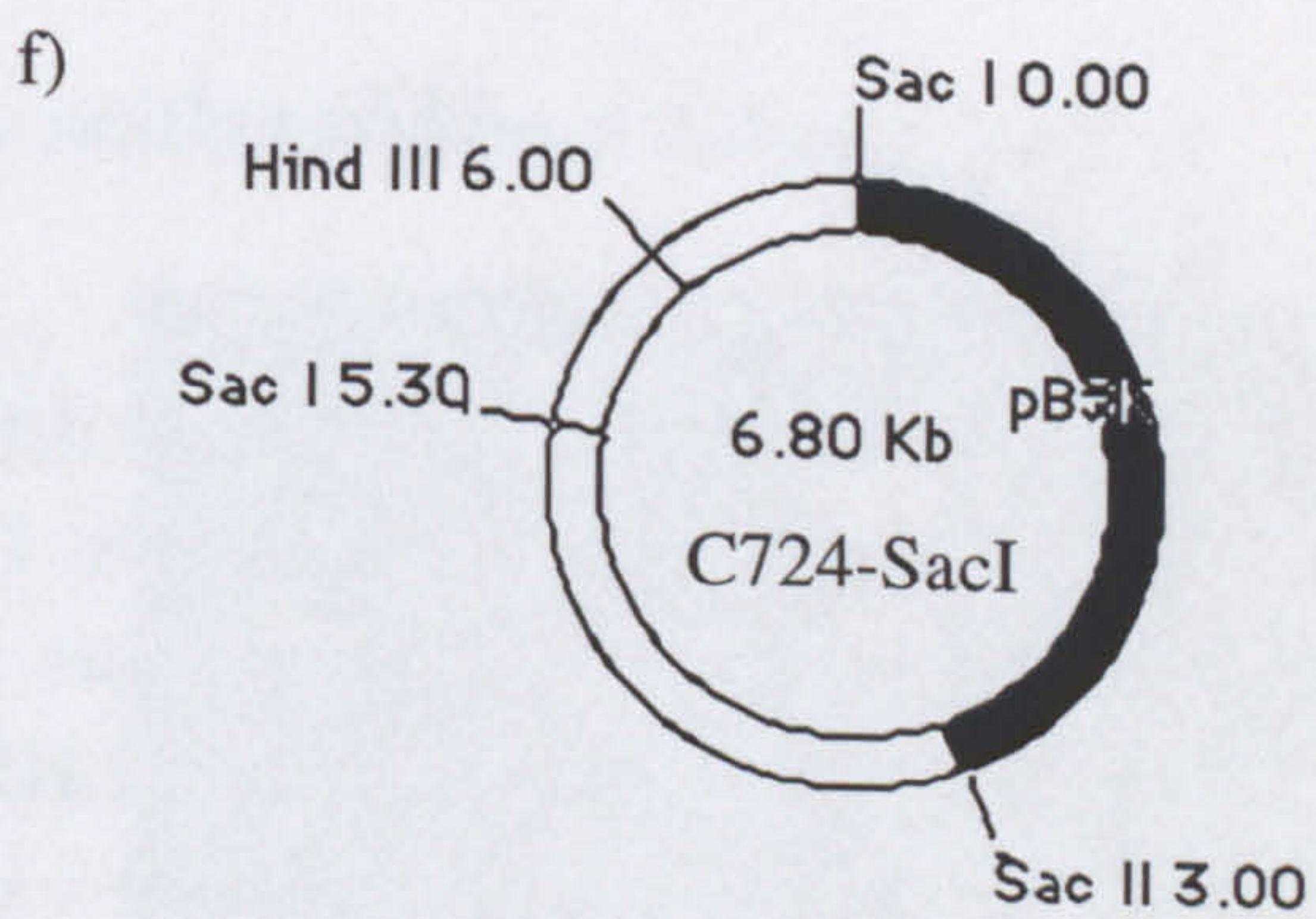
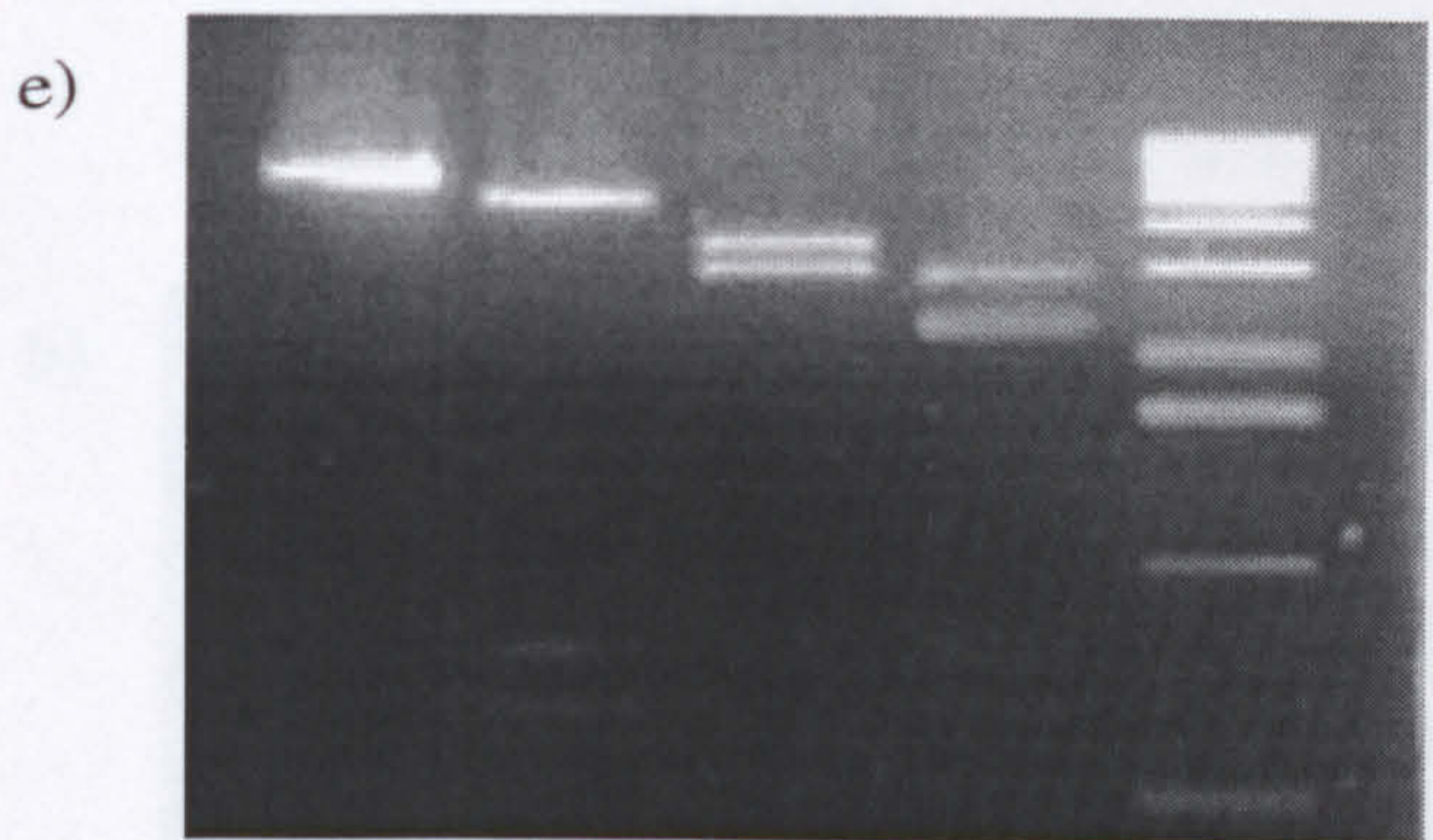
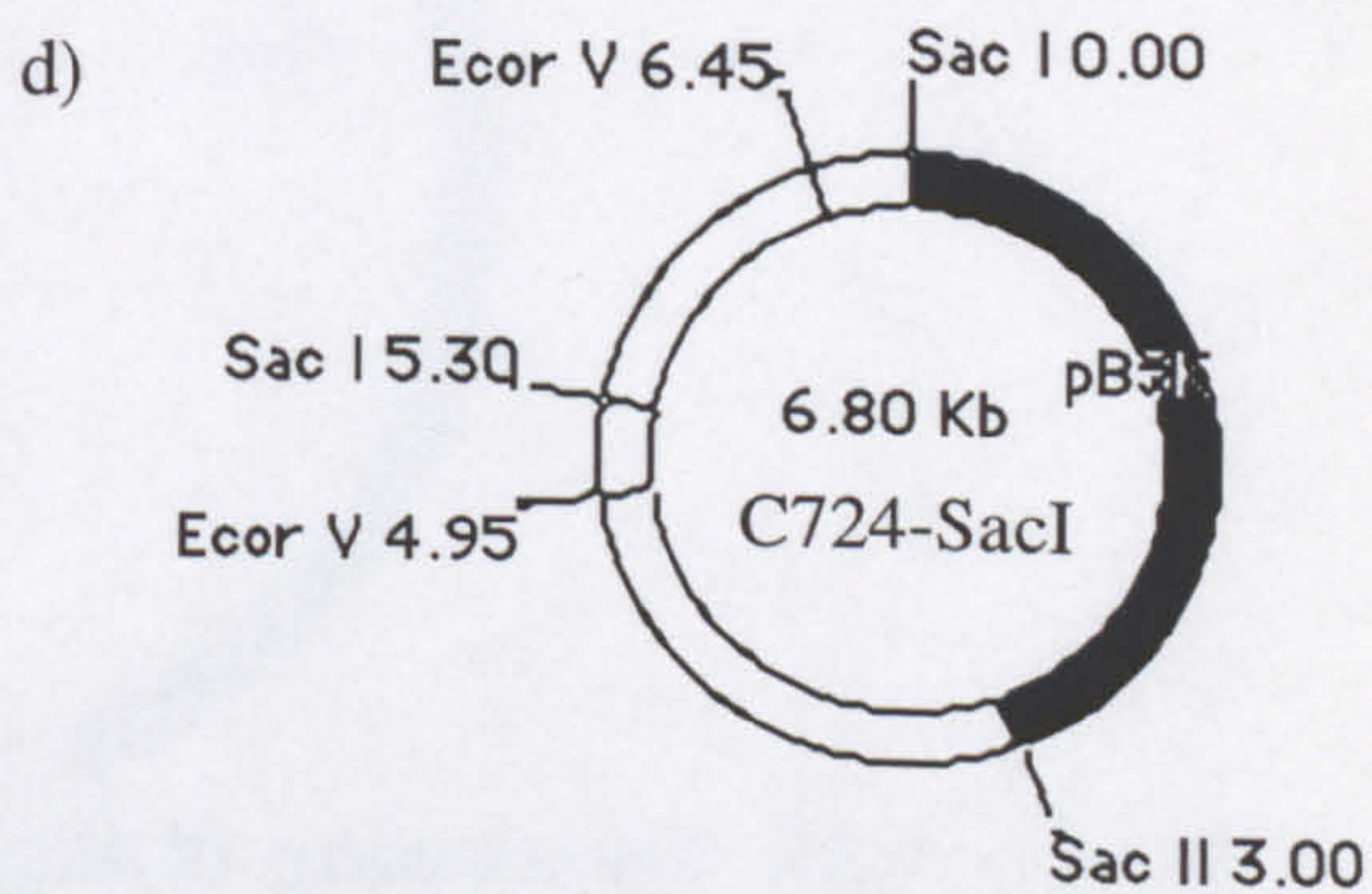
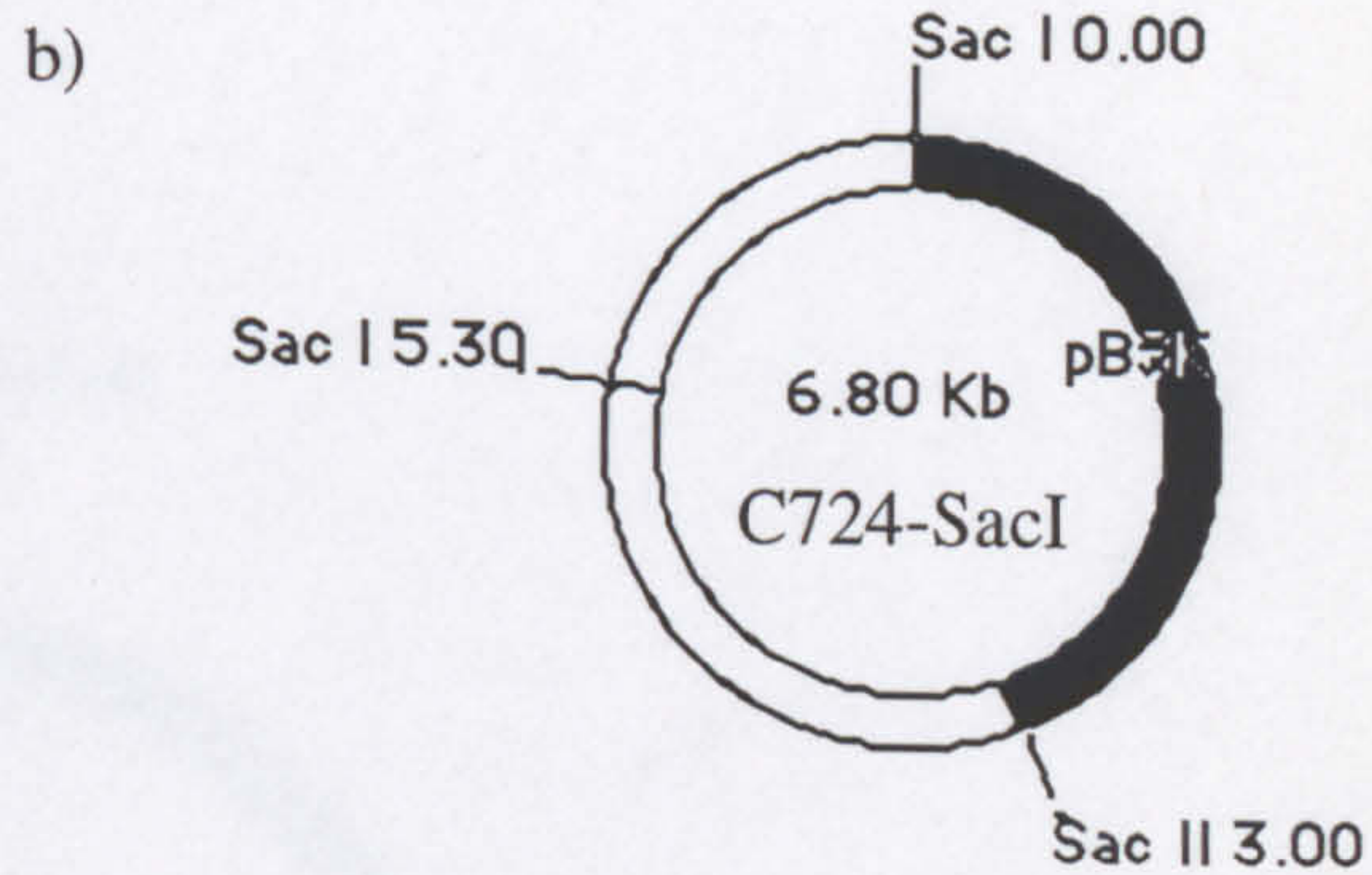
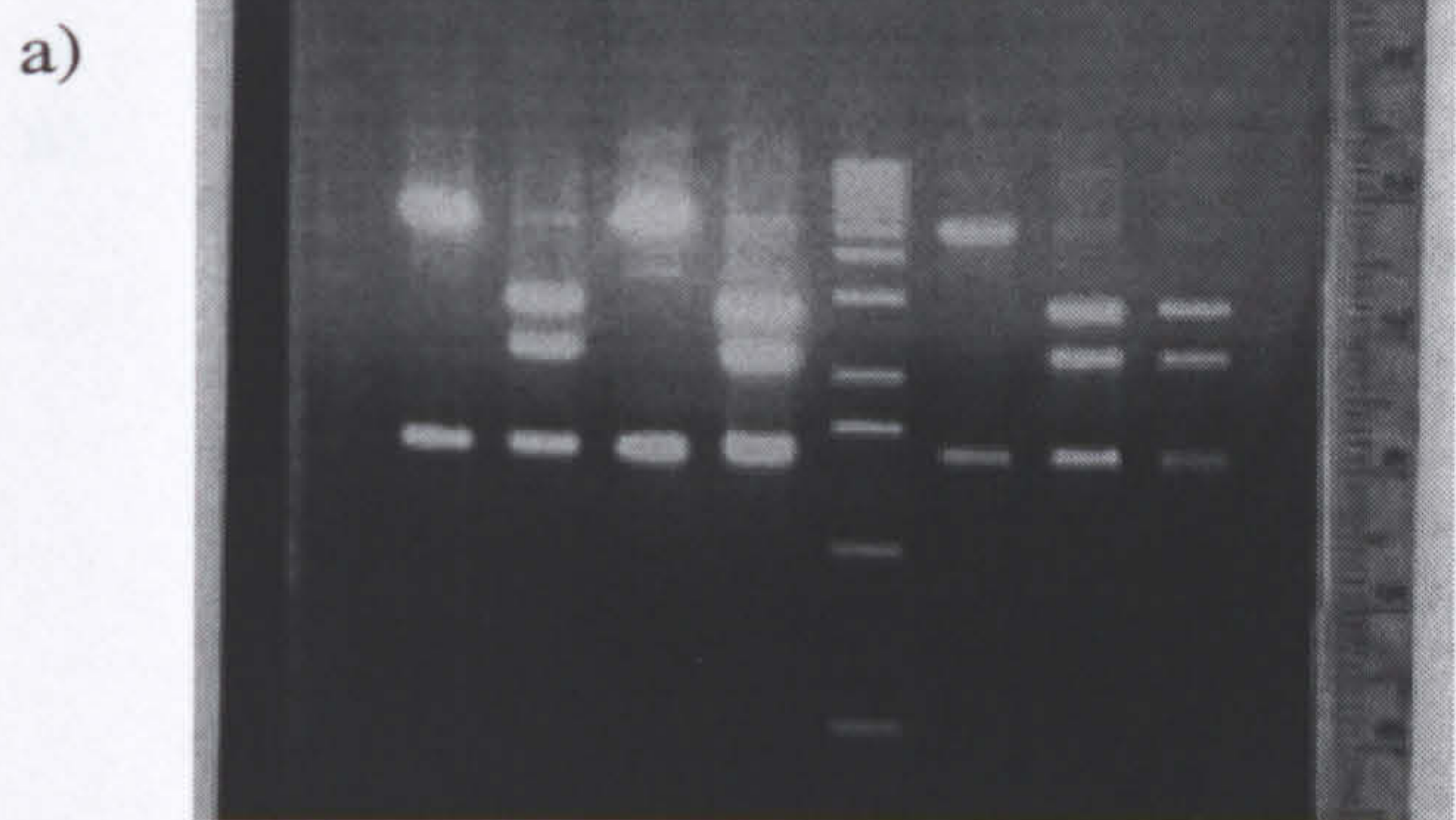


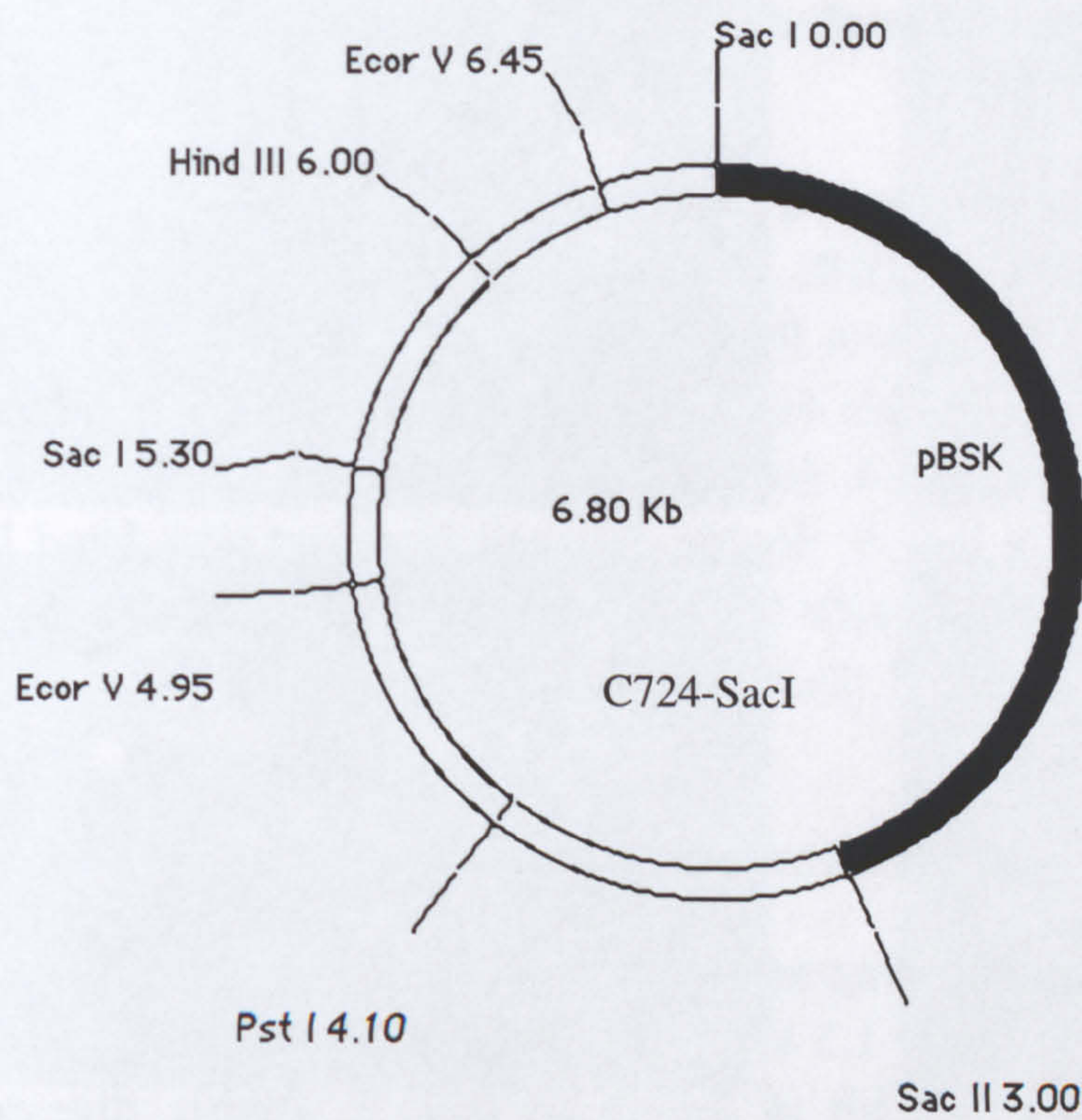
Figure 3.36. Subcloning of digested fragments into pBS from line c724.

a) The schematic representation of restriction mapping to be subcloned into pBS.

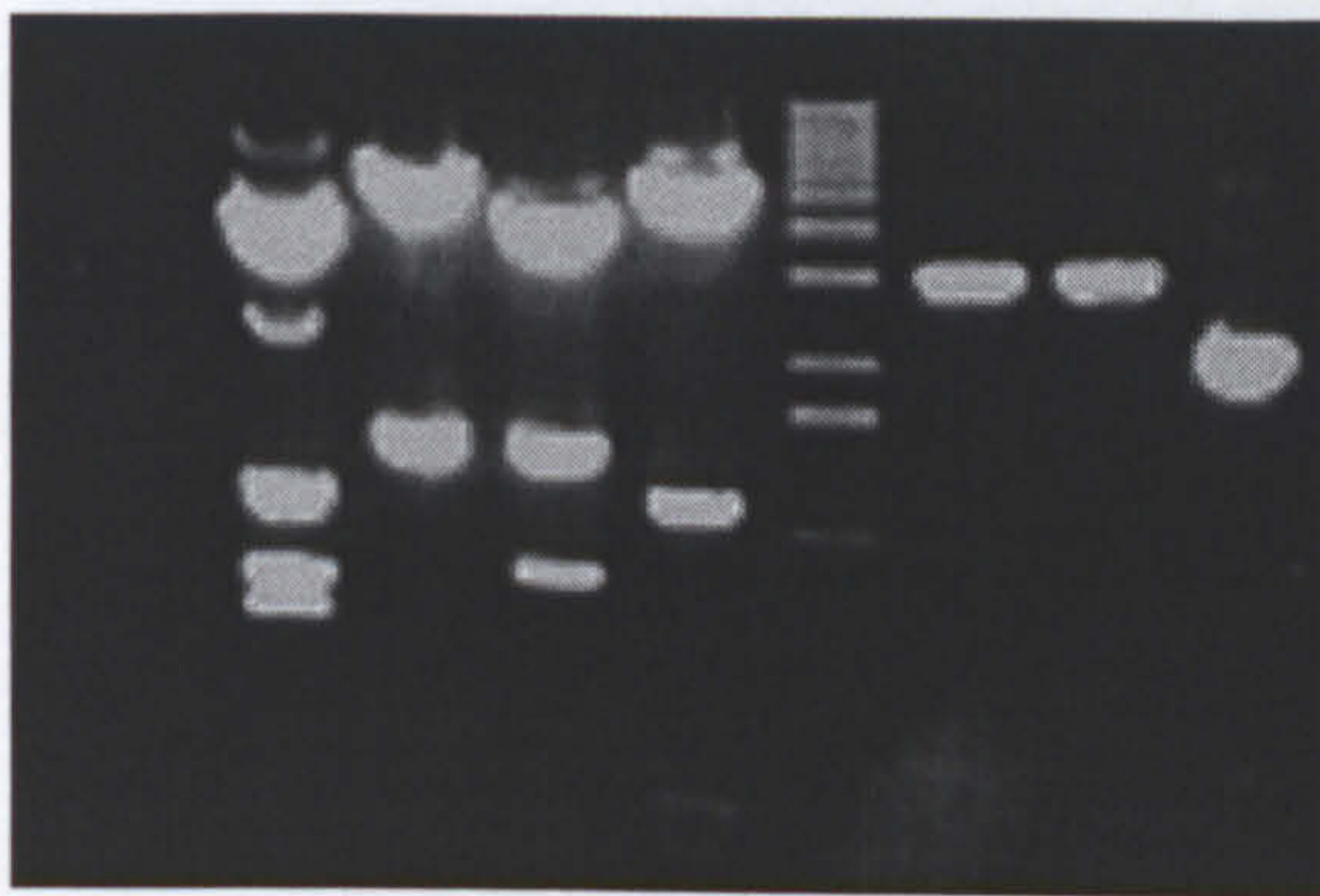
b) The restriction digests with different enzymes before the band cut out (triple digest with Hind III, Sst I and Pst I in lane 1, single digest with EcoRV in lane 2, double digests with EcoRV and Pst I in lane 3, double digests with EcoRV and Hind III in lane 4, standard 1 kb DNA ladder (BRL) in lane 5) with standard 1 kb DNA ladder (BRL) in lane 5).

c) The digested fragments by several enzymes after band excision. Single digested fragment with EcoRV, 1.5 kb in length, in lane 1; single digested fragment with Sac I, 1.5 kb in length, in lane 2; double digested fragment with Sac I and Pst I, 1.2 kb in length, in lane 3; double digested fragment with EcoRV and Pst I, 900 bp in length, in lane 4; standard 1 kb DNA ladder (BRL) in lane 5, double-digested fragment with EcoRV and Hind III, 1.1 kb in length, in lane 6; double digested fragment with Hind III and Sac I, 800 bp in length, in lane 7; double-digested fragment with Hind III and Sac I, 900 bp in length, in lane 8).

a)



b)



c)

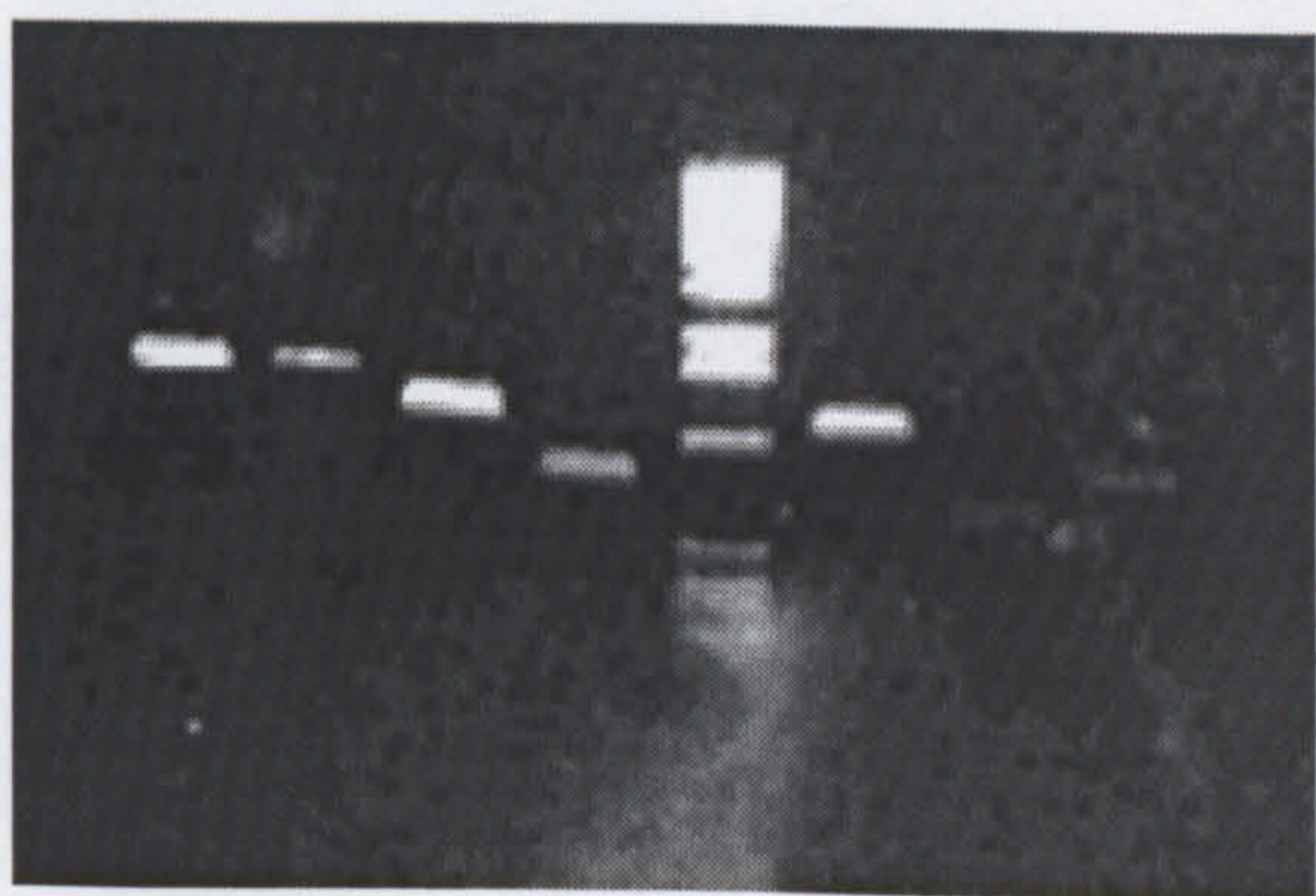


Figure 3.37. Southern blot analysis of subcloned fragments of rescued plasmids from line c724.

a) Some of the other subcloned fragments before Southern blotting (standard 1 kb DNA ladder (BRL) in lane 1, c724 1A (another colony rescued with Pst I) digested with Pst I in lane 2, c724-Pst I digested with Pst I and Sac II in lane 3, double digested fragment with EcoRV and Hind III (1.1 kb in length) in lane 4; double digested fragment with Sst I and Pst I 1.2 kb in length in lane 5, double digested fragment with Hind III and Sst I 800 bp in length in lane 6, double digested fragment with Sst I and Sst II as control in lane 7, single digested fragment with Sst I 1.5 kb in length in lane 8).

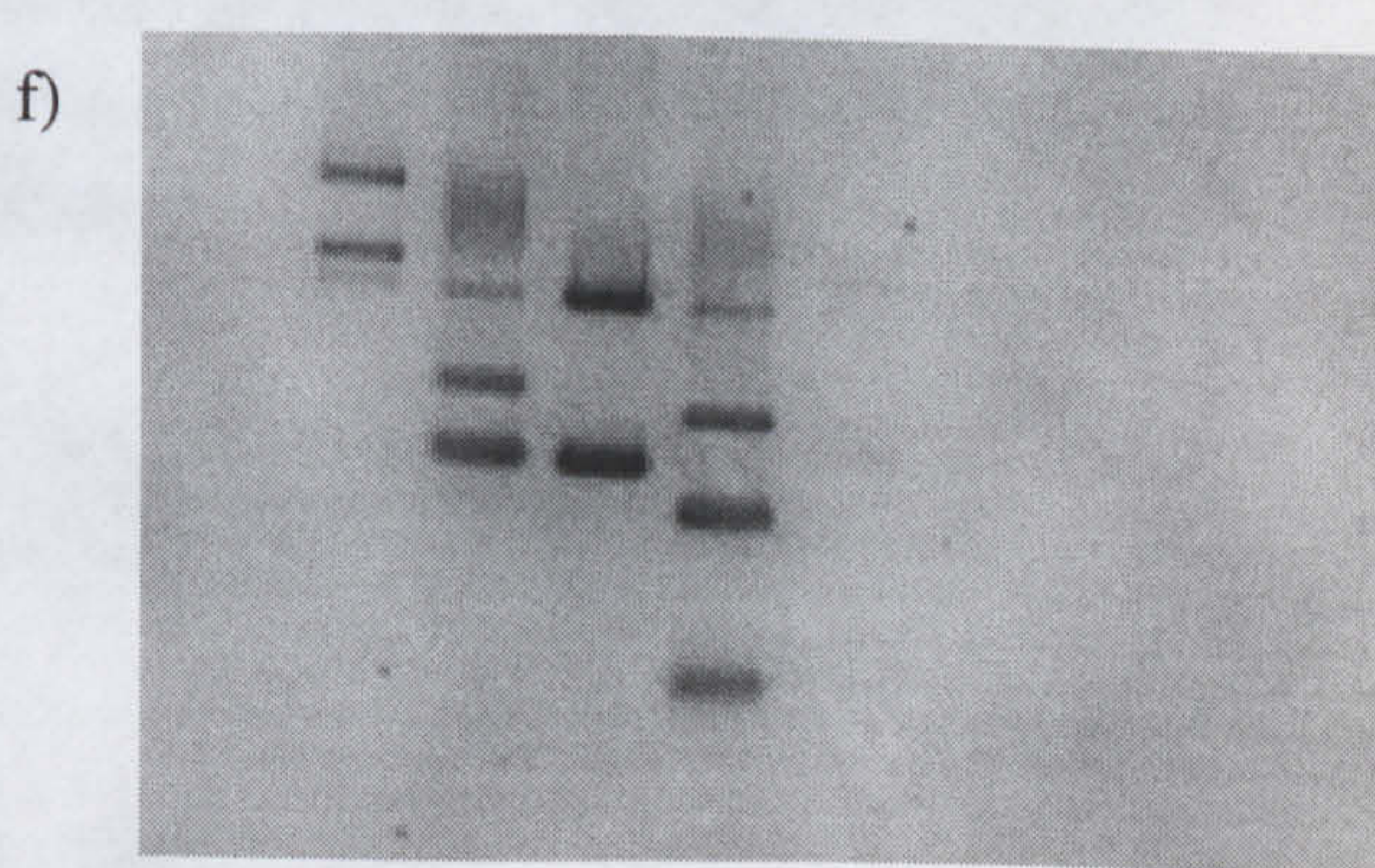
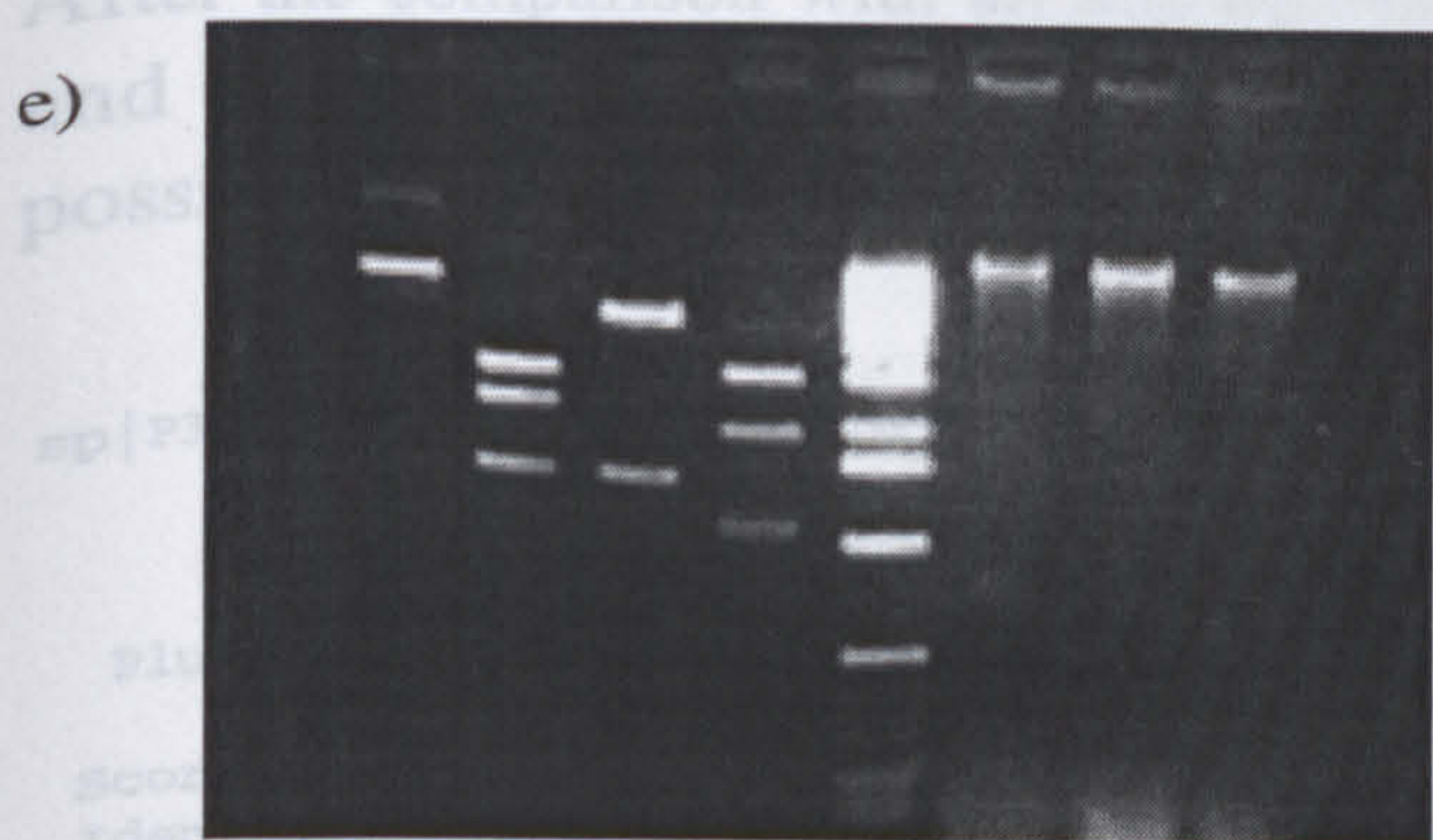
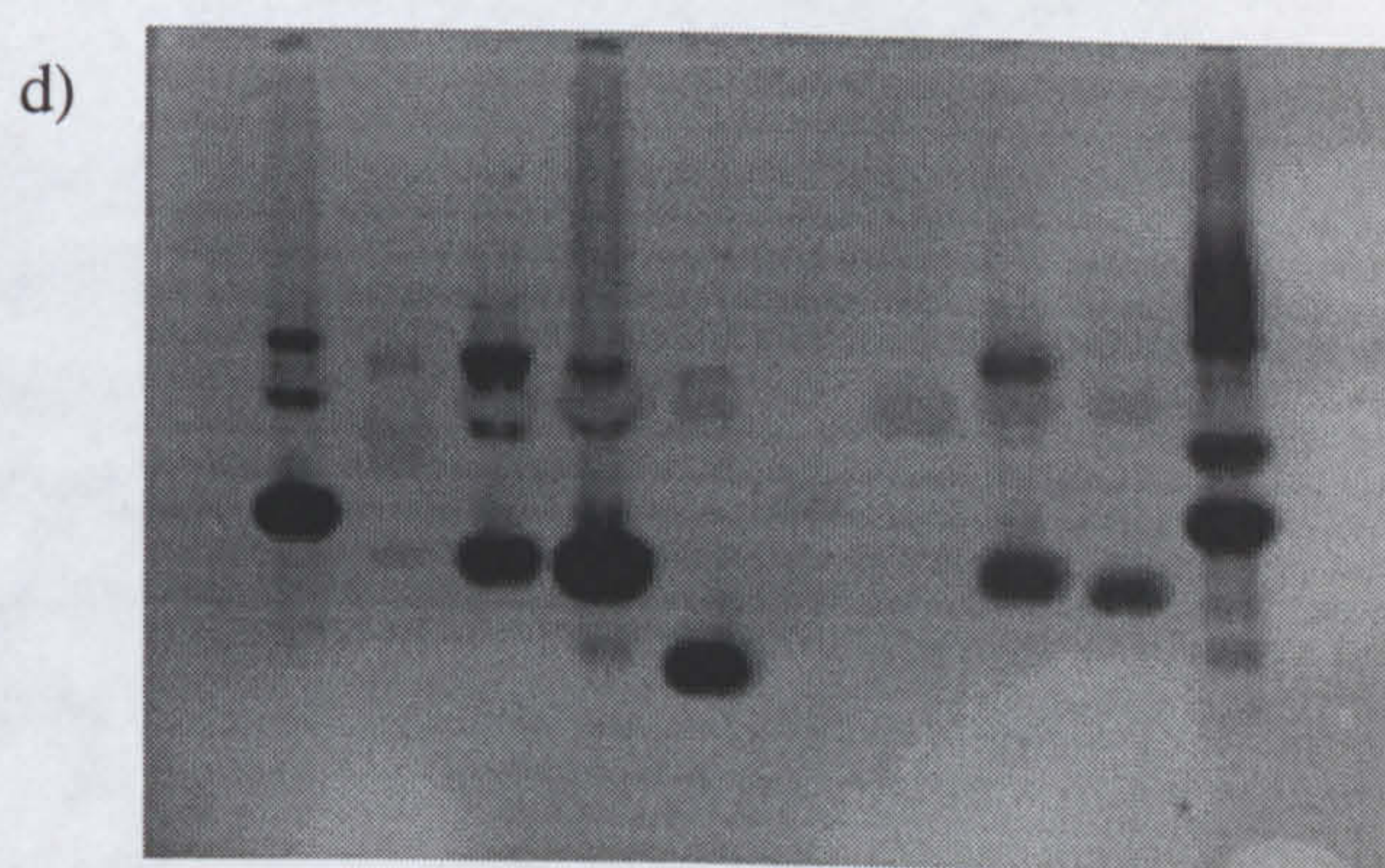
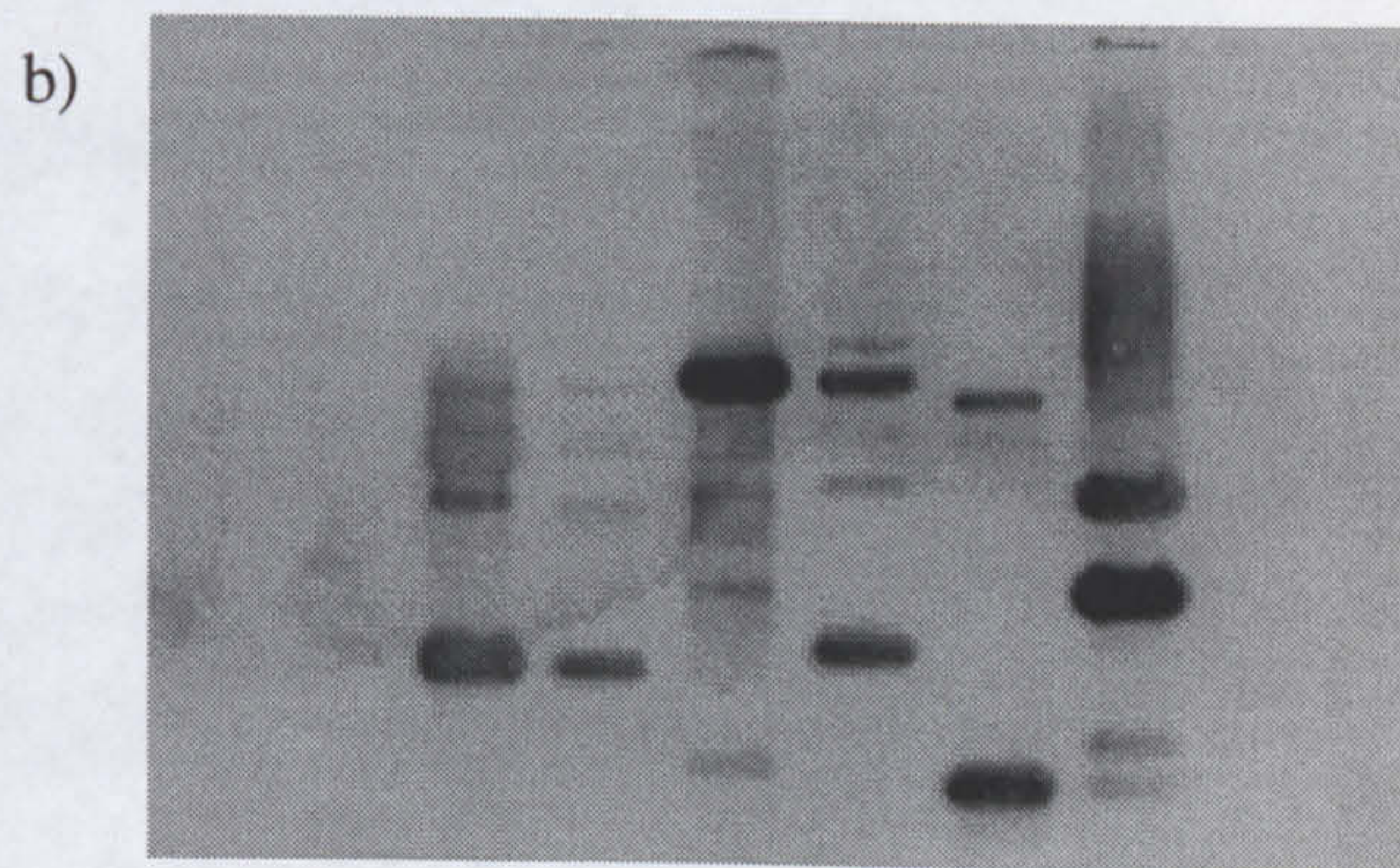
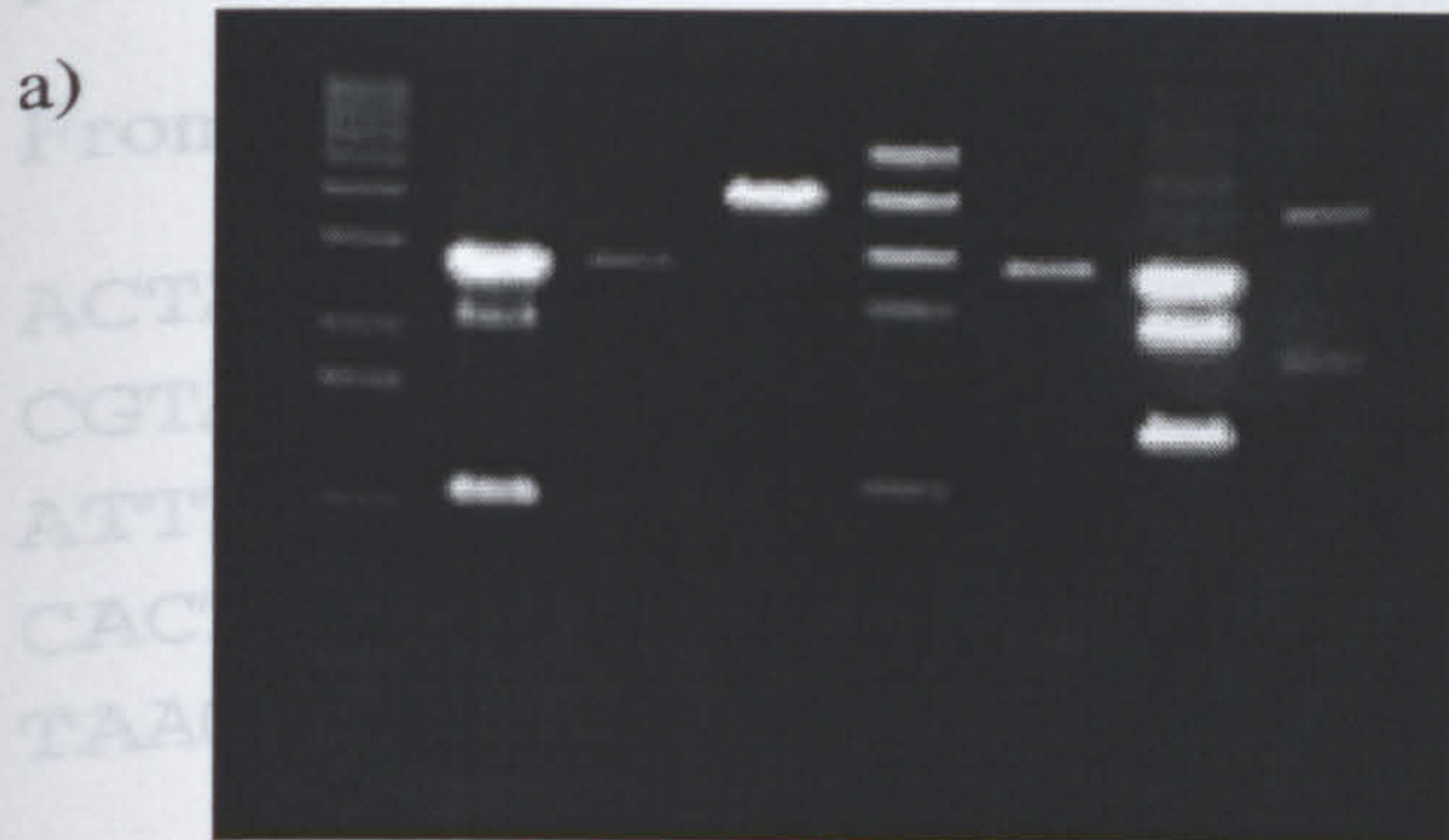
b) The same fragments after Southern blotting.

c) Some of the subcloned fragments before Southern blotting (single digested fragment with Sst I 1.5 kb in length in lane 1, single digested fragment with EcoRV 1.5 kb in length in lane 2, double digested fragment with Sst I and Pst I 1.2 kb in length in lane 3, double digested fragment with EcoRV and Hind III 1.1 kb in length in lane 4, double digested fragment with Hind III and Sst I 800 bp in length in lane 5, standard 1 kb DNA ladder (BRL) in lane 6, double digested fragment with Hind III and Sst I 900 bp in length in lane 7, double digested fragment with Pst I and Sst II 1.1 kb in length in lane 8, double digested fragments with Pst I and Bam HI 1.1 kb and 900 bp in length in lane 9, double digested fragment with Sst I and Sst II 1.5 kb and 2.3 kb in length as a control in lane 10).

d) Some of the same subcloned fragments after Southern blotting.

e) Two of P1 clones before Southern blotting (P1 clone of DS00218 digested with EcoR I in lane 6 and P1 clone of DS03041 digested with EcoR I in lane 7 and 8).

f) The same P1 clones after Southern blotting.



3.8.1. Sequencing and sequence analysis

The flanking sequence of two stellate cell specific lines, C724 and C710 was determined using universal primers to read into flanking DNA from the rescued plasmid. As a result of sequencing the following data were obtained :

From Line C724, rescued with Pst I, and reading away from P-element:

ACTATAGGGCGAATTGGAGCTCTTTGCATGATGTGCTGCCTCCCGCTCTTGGGTCG
CGTATTATTCCGCTTGGCTAATAGAATTACACACAACGCCAAAGATTTATGGGCCC
ATTTAACACGCTTTTGCATTATATTCTCACCCATTTGGGCCTGTTTCATTGATTTTA
CACTGATTAACACGAGCTTAAATTATTCATATTTTTTTTTTTGTCCCGCTTTATAAA
TAACGCATAAATAATAATAATATCGCGACAGCAGCCAAGCCACAATCCT

From Line C710:

CGAGATTTCTAAGGTTATCGCTGTAAAAGTAATGCTAGAAATGGCAAGAAGACCTGA
CGTTCCTAGCATTAGTGAAATGGTGGCACGGACGAGGTTAATGGTTATAACTGGAC
TTAACGGAGCAAATCACCGAAAGGTCCACGTGCATGATGCCTAGAGGCATCGGGAA
ATTGACAGCATGAGGGTTGTGTTAGCGACCTCTGAGGATGGAGAGCGTAGGGCGGT
TGAAGCCAAGGACATTCAATGCCGTCAACGAAATGTCACGGCCAGCACAGTAAGCT
CTAACGTCGTAACCGTCACGTTAGACTCTCGGACCAAACGAAACGTCAATCACAC

After the comparison with all known Genebank sequences at both nucleotide and deduced amino-acid levels, no convincing match was obtained, except possibly for a putative G-protein coupled receptor:

sp|P34972|CB2R_HUMAN CANNABINOID RECEPTOR 2 (CB2) (CX5). gi|407807
 (X74328) CB2 (peripheral) cannabinoid receptor [Homo sapiens]
 prf||1920360A peripheral cannabinoid receptor [Homo sapiens]
 Length = 360
Plus Strand HSPs:

Score = 64 (29.4 bits), Expect = 4.9, P = 0.99
Identities = 13/49 (26%), Positives = 26/49 (53%), Frame = +1
Query: 1 TIGRIGALCMMCLPLLGRVLFRLANRITHNAKDLWAHLTRFCIIFSPI 147
 T+G + A+ ++C P+L + LA ++ K +A + C+I S +
Sbjct: 246 TLGLVLAVLLICWFPVLALMAHSLATTLSDQVKKAFCSMLCLINSMV 294

Chapter 4: Discussion

4.1. Regional diversity in the tubules

It is clear from the results described above that the tubules can be subdivided further than would be possible from either morphological or physiological data alone. Physiological analysis suggests a subdivision into three regions, namely an initial, non transporting part unique to the anterior pair of tubules, a main segment which produces the primary urine, and a lower tubule section which is reabsorptive (O'Donnell and Maddrell, 1995). However, detailed morphological analysis had suggested a subdivision into four regions, namely an initial segment, a main segment, a transitional segment joining these two regions together and a short ureter (Wessing and Eichelberg, 1978). Taken together, the lower tubule region is indistinguishable by morphological techniques and the transitional segment is indistinguishable by physiological techniques. However, both subregions are resolved by the genetic (enhancer trap) approach respectively despite the fact that scope of the screen is not saturating. This shows the power and utility of this approach.

Previously, it had been reported (Wessing and Eichelberg, 1978) that the initial segments of larval anterior tubules were lost during the moult. This is conspicuously not the case in our experience; the anterior tubules of *D. melanogaster* have a prominent dilated initial segment with white material, presumably the concretion bodies reported in larvae (Zierold and Wessing, 1990), in the lumen. In these respects, as in the relative length of anterior and posterior tubules (see below), the adult tubules described here resemble most closely the classical description of larval tubules (Wessing and Eichelberg, 1978), a view shared by Janning *et al.* (1986). The reason for this is unclear.

The boundary between initial and transitional segments is confirmed by enhancer trap studies, with complementary lines available that mark the boundary from opposite directions. Surprisingly, it has been found that these lines also stain the tips of the posterior tubule pair, in which initial segments are not morphologically apparent. Furthermore, by counterstaining nuclei with ethidium bromide, it can be stated that this compartment is 7 and 44 principal cells long in posterior and anterior tubules respectively. This implies that the anterior and posterior tubules

differ in extent rather than in nature (qualitatively). They can also be considered to comprise analogous functional compartments (non secretory combined initial and transitional regions) as suggested either by ethidium or rhodamine 123 transport assay respectively (**figs 3.5 c and 3.6**). It is thus clear that, both genetically and functionally, both anterior and posterior tubules possess both initial and transitional segments.

The transitional segment might be considered to be merely an area in which the properties of initial and main segments merge seamlessly, and the transitional segment is classically described as being relatively short compared with the initial segment. However, evidence from gene expression suggests that the transitional segments, rather than merging seamlessly, have a distinct genetic identity; that is, they constitute a distinct segment. Lines can be found (C825) in which the staining stops at either end of the transitional segment, rather than smearing across it. Nuclear staining suggests that this is 22 principal cells long, equal to the initial segment. The region is also apparent genetically in posterior tubules and 3-4 principal cells long, although it has not previously been reported there. Given this difference with classical studies, it could be argued that our “transitional segment” is not the same as that previously described. However, we found no other subregion between initial and main segment, and the main segment is active both in ion transport, fluid secretion and dye transport; so our assignment of this segment is plausible.

The main segment starts below the transitional segment, and runs to around 20% of the tubule length, based on cell counts. There is convergence here between the morphological and genetic studies, together with the lower-resolution mapping of the physiological studies and the V-ATPase reporter gene expression.

The lower tubules, which are reabsorptive in nature (O'Donnell and Maddrell, 1995), can also be subdivided into multiple regions and show slightly different structure from the main segment (Wessing and Eichelberg, 1978). The boundary between main and lower tubule is again supported by the evidence from gene expression (lines C507, C232), but here again the latter technique can provide a higher resolution of the lower tubule into two distinct regions. The ureter has been found to be subdivisible into two subregions as upper and lower ureter reported by

two independent lines, C649 and C601 confirming the classical description in terms of the organisation of their cells (Wessing and Eichelberg, 1978).

It has been possible to reconcile these boundaries with a range of physiological markers, such as the transport of inorganic ions, organic dyes and fluid; and with histochemistry for other enzymic activities, such as alkaline phosphatase. It seems then, that the enhancer-trap lines assist our discovery of genuine biologically distinct domains in this tissue as can be seen in detail in section 3.5.

4.2. Cellular diversity in the tubules

Previous reports identified two major cell types in tubules (Wessing and Eichelberg, 1978): a principal, type I cell and a rarer type II or “stellate” cell. The principal cell is a classic squamous epithelial cell with microvillate apical border and involuted basal border, whereas the minor stellate cell shows a cruciform morphology which appears to “slot in” along the interstices between principal cells. There is also a clear dichotomy between the structure of the type I and type II cells of the initial segment and those of the rest of the tubule; in the initial segment, the cells are extremely flattened and appear devoid of any ultrastructural complexity (Wessing and Eichelberg, 1978). A combination of painstaking developmental observation and genetic markers has also identified the presence of a neuroectodermal tip cell at the end of each developing tubule, and shown that cells with similar properties persist into larval, though probably not adult, life (Hoch *et al.*, 1994).

Enhancer trap studies bear out these observations but also produce new insights. Although no tip-cell specific lines were observed in this study, they have been observed by others (Skaer and Johnson, personal communication). It seems clear that there is a previously unsuspected heterogeneity even among principal cells of the main segment; that is, the primary cells are also subdivisible, even within the main segment; although some lines stain all the principal cells, others label only the majority, and others cleanly label a small variable minority between 5% and 20% of the principal cell population.

The unique identity of the stellate cells (type II) is particularly striking in this study. Two independent lines (C710 and C724) appeared to stain

only stellate cells within tubules, and furthermore one (C724) appeared to stain nearly nowhere else in the organism in either larvae or adult. The independent identification of the same subset of cells by two independent insertion events reinforces the argument that these represent a distinct subpopulation. Additionally, the staining of bar-shaped cells in the initial and transitional segments (**fig 3.5 c**) of the tubule implies that these are, despite their different morphology, homologous. In support of this, line C709 which marks them all by exclusion (**Fig. 3.16**) suggest that they should be homologues. However, line C42 marking the bar-shaped ones by inclusion and stellate-shaped ones by exclusion suggest that they could be different subsets of cells in morphologically and functionally different regions (**Figs 3.15, 3.21**) as in line C649 which marks only bar-shaped cells by inclusion (**Figs 3.14, 3.19**). In summary, although there is a clear dichotomy in the two subtypes of stellate cells in morphology, they could undertake a similar but at present unknown function in the distinct (secretory and non-secretory) functional main subregions as described for two subtypes of intercalated cells in the medullary and cortical collecting duct of rat kidney (Alper *et al.*, 1989). Enhancer trapping also makes it possible, for the first time, to demonstrate that their spacing along the length of the tubule is approximately even, suggesting that they may differentiate by a process of lateral inhibition (Lawrence, 1993).

The role of stellate cells is controversial; they have been argued to act as chloride-transporting cells (O'Donnell *et al.*, 1996; Pannabecker *et al.*, 1993), or to reabsorb solutes from the urine (Meulemans and De Loof, 1992). It is clear that they are unlikely to pump protons like the principal cells, as they do not stain for V-ATPase-directed reporter expression (**Figure 3.6g**). It has also been found that they stain immunocytochemically for BLIP, a member of the MIP family of proteins which include water channels (Dow *et al.*, 1995). A general role in urine production is rendered less likely by the fact that many orders of insects appear not to have stellate cells; however our prediction is that in such species, the role of the stellate cells may be taken by an as yet unidentified subpopulation of principal cells.

The study also demonstrated the presence of a previously unreported cell type in the lower tubules (**Figs 3.3 g, h**). These cells are very much smaller than any others noted in this study, having a reduced cytoplasm around a compact nucleus. The likeliest roles for small cells of this kind

in insects are thought to be neuroendocrine, immune or regenerative. At present, it can be speculated that these are neuroendocrine, based on their staining for HRP. However, now that these cells have been marked genetically, further elucidation of their role should be easier.

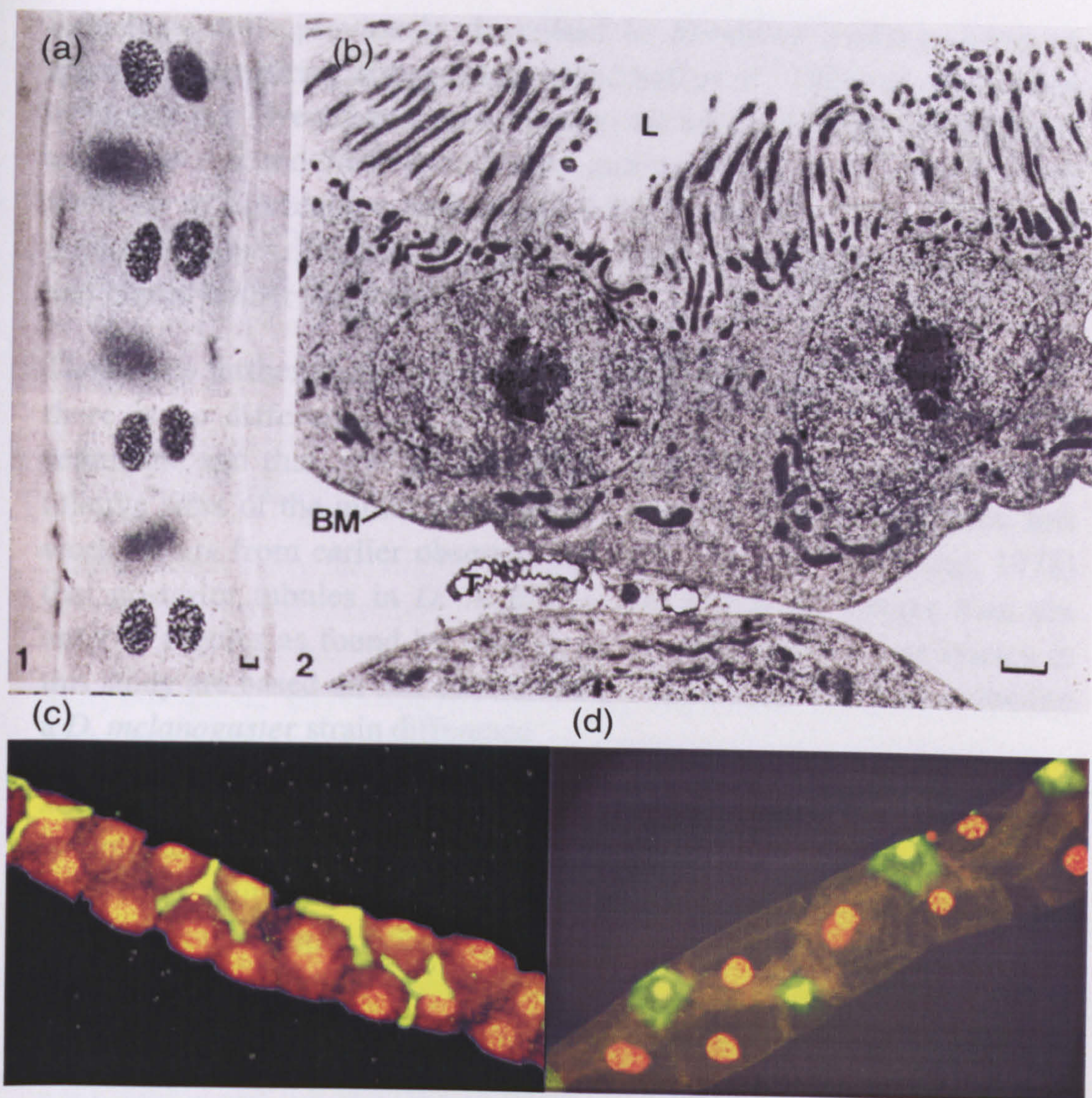
4.3. Quantification of tubule domains and cell types

GAL4-expressing tubules were counterstained with ethidium bromide to reveal cell nuclei. Distance between expression boundaries were measured by counting cell nuclei from either end of the tubule except ureter. It was possible to establish a numerical map of the tubule domains and cell types defined by gene expression. The data obtained by this method are comparable with the previous studies on the embryo consisting of 2x140 AMT cells, 2x 105 PMT cells (Skaer and Martinez-Arias, 1992) and on larvae consisting of 490 cells (2x141 AMT cells, 2x 104 PMT cells and on adult consisting of 484 cells (2x136 AMT cells, 2x 106 PMT cells (Janning *et al.*, 1986) shown in **Figure 4.1**. However, our values (145 ± 1 and 111 ± 1 in the anterior and posterior tubules, respectively in adult and 135 ± 1 and 104 ± 1.4 in the anterior and posterior tubules, respectively in third instar larvae) are solely for principal cell nuclei and exclude approximately 33 or 21 stellate cells in anterior and posterior tubules, respectively. How could these data be reconciled?

There could be several possibilities:

- (1) One or other set of data is in error.
- (2) There are differences between *D. melanogaster* strains.
- (3) At some stage in development, principal cells become binucleate.
- (4) Stellate cells arise relatively late in development, and their smaller nuclei leads to their being easily overlooked.

We cannot exclude the first possibility, although all studies appear to have been painstaking. Similarly, we cannot exclude the second possibility, although all experiments seem to have been performed on standard strains such as Oregon R and Canton S. The third possibility merits further discussion; however, in this study, it does not seem to be likely, because inspection of the cells shown in **Figure 4.1** suggests that each principal cell seems to contain only one nucleus. By contrast, the



(e) Total cell numbers in the Malphigian tubules

STAGE	AMT	PMT	MT	REFERENCE
Embryo	140	105	490	Skaer, <i>et al.</i> , 1992
Larvae	141	104	490	Janning <i>et al</i> , 1986
Adult	136	106	484	Janning <i>et al</i> , 1986
Larvae (3)	135	104	478	Sozen <i>et al</i> , 1996
Adult	145	111	502	Sozen <i>et al</i> , 1996 (submitted)

Figure 4.1. Comparison of cell numbers in the Malphigian tubules
 (a) Binucleate cells from *Rhodnius prolixus* (Maddrell, *et al.*, 1985). (b) GAL4 expressing stellate cells and ethidium stained principal cell nuclei in adult and in larvae from line C724 (d). Comparison of cell numbers of Malphigian tubules in embryo, larvae and adult and its distribution to the anterior and posterior tubules.

binucleate cells previously described in *Rhodnius prolixus* have an unambiguous pairing arrangement (Maddrell *et al.*, 1985) as can be seen in **Figure 4.1**. Even if this were the case, the totals of tubule cell numbers would be 106 and 74 in the anterior and posterior tubules respectively from tip to bifurcation, and would differ even more from previously published values. For now, the likeliest possibility seems to be the fourth, and merits further analysis.

These data further show that, between the anterior and posterior tubules, there is no difference in cell numbers in the main or lower tubule segments, and that overall differences are entirely attributable to the relative sizes of the initial and transitional segments. In this aspect, this work differs from earlier observations (Wessing and Eichelberg, 1978) that posterior tubules in *D. melanogaster* adults are longer than the anterior tubules as found by Janning *et al.*, (1986). As the estimates in this study are based on cell counts, the previous data must be ascribed to a *D. melanogaster* strain difference.

Although the reconciliation of genetic and functional maps and complementary boundaries reported in different lines (**Figs 3.5 c, 3.10 a, 3.10 b**) is quite precise and confirmed statistically, there are minor differences that merit explanation. For example, the very slight difference between the number of ethidium-stained principal cells (40 ± 0.6) in the compartment where the bar-shaped cells are localised (marked by line C649) and the total number of principal cells in the combined initial and transitional segments marked by line C568a (44 ± 1.7) and line C42 (45 ± 1.7) in adult, could have arisen because of the nature of the boundary marked by line C649. That is, counting of principal cells stops at the most proximal bar-shaped cell; however, if the true boundary lies alongside the first stellate cell (i.e. the first type II cell not included in line C649), then line C649 could faithfully mark a compartment while providing an underestimate of true cell number. However, this difference is very small, and in larvae, the difference between values is not significant (**Figure 3.10 a**).

Similarly, there is a small discrepancy in adults between the numbers of principal cells in the compartments of anterior tubules excluded from staining by alkaline phosphatase, and the line C507 that marks the alkaline phosphatase gene (**Figure 3.5 d**). However, we ascribe this

difference to a biased mean of 118 cells for line C507, which contained an unusual number of extremely large tubules. Indeed, the values in larvae agree perfectly both with each other, and with the alkaline phosphatase-marked compartment in adults, at 108 cells (**Figure 3.10 b**).

It was also possible to distinguish nuclei of principal, stellate and "tiny" cells on the basis of nuclear size. This can serve as an objective method of congruence between boundaries in different lines and allows their subsequent study independent of reporter gene marking.

4.4. Relationship between domains of gene expression and functional boundaries

These results allow the exploitation of the simplicity of structure of the tubules to bring to bear multiple experimental techniques on the question of cell type and identity in a differentiated tissue. In all cases, the morphological, physiological and immunocytochemical boundaries identified can be aligned with enhancer-trap marked genetic boundaries, providing a useful mutual validation of both techniques. However, analysis of enhancer driven gene expression provides further evidence of richness in this tissue; there are at least two new regions (initial segment of posterior tubules and subdivision of lower tubule) and two new cell types (subdivision of principal cells and small cells of lower tubule) implied by these results. Additionally, the markers of stellate cells allow us to infer a genetic similarity between the stellate-shaped cells of the main tubule and the bar-shaped cells of the initial segment, a similarity which might be overlooked on morphological grounds alone.

In this particular tissue, the results provide exciting opportunities for further work on the developmental specification of these subtypes, and on the physiological processes mediated by these multiple genetic domains defined by gene expression. In more general terms, the combination of genetic and other techniques should allow us to validate further the principal of inferring functional boundaries from those reported by enhancer detectors within a genome.

4.5. Aspects of stellate cell identity revealed by developmental P{GAL4} expression patterns

The developmental analysis of stellate cell specific lines confirms the significance of these population of cells strikingly. So stellate cells should be one of the major cells of Malpighian tubules. Although the enhancer trap data implied similarity between the stellate-shaped cells of the main segment and the bar-shaped cells of the initial and transitional segment, they were clearly distinct morphologically, a finding supported by the bar-shape-specific line C649. Developmentally, this distinction is reinforced. In two different lines, C724 and C710, although P{GAL4} is switched on in all stellate cells in the adult, in line C710 only main segment stellate cells are switched on in larval stages suggesting the diversity between these two subsets, at least in some sense as observed by Alper *et al.* (1989). In addition, line C649 with its expression pattern confined to bar-shaped cells of both larvae and adults, confirms the diversity.

The very close figures obtained by calculation of respective numbers of the total stellate cells in third instar larva from these lines (C649 v C710) are complementary to each other (Table 3.3). This reinforces the notion of genetic diversity, and it is conceivable that the two subtypes of cells could be distinct, but sharing some similarity of morphological and functional properties. From the classical studies, the proximal and distal parts of the tubules were considered to be different functionally from one another (Bradley, 1984; Wessing and Eichelberg, 1978). Although there is a clear dichotomy in the two subtypes of stellate cells in morphology, they could undertake similar function in the distinct (secretory and non-secretory) functional main subregions as described on two subtypes of intercalated cells in the medullary and cortical collecting duct of rat kidney (Alper *et al.*, 1989) even developmentally.

Since the number of stellate cells through all developmental (larval) stages analysed remain unchanged, it confirms that after the proliferation finishes at embryonal stage, only the size and the shape of the cells differentiate and rearrangement occurs. The relative number of principal and stellate cells obtained here presents new evidence for this process (Skaer and Martinez-Arias, 1992).

4.6. Other aspects

This study shows some of the uses of enhancer trap technology. Of these, the development of vital staining techniques allows the combination of

physiological techniques with the genetic and molecular. So, on the genetically marked subregions, distinct cell types and even subsets of cell types can be dissected for further characterisation which was not possible on the fixed tissues. The physical isolation of single genetically defined cells can give extra advantages to characterise them at the molecular level, for example by subtractive hybridisation techniques.

GFP, besides saving a lot of time in visualisation in comparison with histochemical methods, also suggests some vital manipulation of this genetically marked and compartmentalised tissue. The observation of a range of shapes in stellate cells (**Figs 3.24, 3.25**) could have several underlying causes. Obviously, one apparent reason is developmental stages; stellate cells seem to be much more round-shaped in larval stages not stellate-shaped as in the adult particularly the bar-shaped ones. A second possibility could be that the stage of urination affects cell morphology, as sometimes they seem to differ in shape even at the same developmental stage, such as in the adult in **Figure 3.12 g,h**. As it has been proposed that chloride and water flux is through these cells (O'Donnell *et al.*, 1996), there may be stellate cell-specific transient volume changes associated with these changes in permeability (Rothstein, 1989; Kersting *et al.*, 1993). Vital marking of stellate cells with GFP may allow real-time confocal 3D reconstructions of stellate cells throughout the course of hormonal stimulation.

The GFP findings also confirm that the ureter is subdivisible into two regions which could be called as upper and lower ureter even developmentally. Two independent lines C507 and C42 (**Figs 3.20, 3.21**) mark the upper ureter by inclusion and lower tubule by exclusion in larval stages (third instar), although both of them mark all the ureter by inclusion in the adult, confirming the mentioned morphological finding. This also suggests that the other data are confirmed by genetic evidence nearly all the time besides the new findings.

Some lines expressing variegation in principal cells were tested in terms of temperature and sex. It appears that temperature has no effect on the variegated expression in the principal cells suggests different from previous results. As a result of these preliminary works, the sex factor seems to have some effect on the variegated expression. However, it remains for further work to prove it. Another factor that could be

considered is the age due to the fact that some lines (like C825) have been observed to show different level of expression, that is, a stronger expression in the 1-2 days following hatching and a weaker expression a week later. So, these lines could also have the same property.

In situ chromosomal localisation allows early identification and preliminary information about candidate genes specifically expressed in the Malpighian tubules. For instance, when the chromosomal location obtained by *in situ* hybridisation is checked for line C855, it is found that that location, chromosome 2R, location 46E, corresponds to the gene encoding the non neuronal form of synaptobrevin (*Syb*). This is a plausible candidate, due to the fact that all cells have vesicles and principal cells have lots of ommochrome-containing vesicles. So, reconciliation of *in situ* localisations with the *Flybase* genome project data may occasionally help to get an idea about the control elements driving the expression of GAL4 even prior to cloning and molecular characterisation.

It was aimed to observe the tubules without stellate cells by ablating them all including bar-shaped ones. Although it has appeared that the stellate cells have not been ablated completely and effectively, at least some degree of damage was observed to some extent by inspection or tubule assay. It suggests that with effective ablation agents it could be more useful and it could show the utility of enhancer trap technique by which a combination of molecular, genetic and physiological techniques can be used at the same time .

4.7. Molecular aspects

Before the second generation enhancer trap technology, the first generation enhancer trap studies mainly served as means for cloning and characterisation of the genes as well as a method for studying of expression pattern.

Potentially, the second generation enhancer trap technique is capable of serving the same purpose, but has other extra advantages. So, the lines screened and identified on the basis of expression patterns is also capable of providing a route to cloning tubule-specific genes. For this, the initial steps taken are plasmid rescue operation and subcloning of rescued

genomic fragments for sequencing and further characterisation at the molecular level.

Following the plasmid rescue operation, all the fragments rescued were found in different sizes confirming their authenticity and different genomic localisations. So, it is not far away that cloning and further characterisation of these very tubule specific genes, expressed even in limited subpopulations such as just the bar-shaped cells and some of the principal cells in the main segment, or just only in a subregion and ureter, is expected to elucidate the functions of their parts in osmoregulation and transport processes together with its control in this transporting epithelium.

For example, cloning of the flanking genomic DNA around the P insertion of line C507, expressed in lower tubule and ureter revealed a transcription unit with homology to alkaline phosphatase (Yang, pers. comm.). Although it is early now to understand the function and mechanism of alkaline phosphatase in this tissue. Future advances should help to uncover its role. Although enhancer detector may not report precisely the expression of the nearest transcription unit, its activation relies nonetheless on a combination of cellular transcription factors and thus reflects the underlying organisation of a tissue.

All the other candidates have the same potential for elucidation of the mechanisms and the roles of compartments as a subregion or subtypes. For instance, "what is the role of stellate cells in this tissue?". The answer can be obtained from lines C724 and C710. "What is the function of the bar-shaped cells and their difference from the stellate-shaped cells in the main segment even developmentally?". Line C649 and C42 could have the answer, as they are expressed uniquely in these cells of anterior tubule not in the stellate-shaped cells of main segment.

Potentially the most interesting genes are those driving expression of the tubule specific lines of C710 and C724. These genes do not share chromosomal loci with any known candidate for stellate cell-specific genes, for example DRIP, the putative water channel at 48F. In fact, Flybase suggests very few candidate genes at the C724 locus.

In addition, the sequence data from the preliminary work on the rescued plasmids has no homologues in the Genbank database at either DNA or

protein levels. Thus, particularly C724 seems to be presumably a new gene specifically expressed only in stellate cells (including bar-shaped ones) throughout all developmental stages from late embryo to adult. So it seems very much likely to present some novel insights about the function and role of stellate cells in development as well as in mature tissue (adult Malpighian tubules). Given the similarities with vertebrate kidney intercalated cells (Alper *et al.*, 1989), it is possible that some of these novel genes may lead to the identification of vertebrate counterparts.

The lines also suggest some interesting questions. “To what extent do the main segment and initial segment and lower tubule and transitional segment have similarities in terms of function?” Line C825 and C507 could give the answer. “What is the reason for the heterogeneity of the main segment principal cells (on the basis of expression)?” Line C324, C855 and many other second generation enhancer trap lines could have the potential answers of these kind of crucial questions which could enlighten us on these compartments’ particular roles in this small model tissue particularly with the combinations of other physiological, morphological and molecular techniques currently available in *Drosophila*.

4.8. General discussion and future implications

Transporting epithelia have a critical role in osmoregulation, and transport processes and their control are subjects of extensive research. A unique model epithelium preparation has been recently developed, in which physiological studies can be combined with biochemical and molecular analysis, but in which the tissue is also amenable to detailed molecular genetic manipulation, using tools unique to *Drosophila* (Dow *et al.*, 1994a).

The renal (Malpighian) tubules in *Drosophila* have been studied extensively from a developmental standpoint, and cell proliferation during embryogenesis has been shown to be promoted by tip cells (Skaer, 1989). Allocation of each tip cell from each primordium is under the control of proneurotrophin and neurogenic genes to those required to specify cell fates of the peripheral nervous system (Hoch *et al.*, 1994). They consist of two epithelial sub-types. It has been shown that it is possible to perform physiological analysis of this tissue, and shown that cAMP

(Dow, *et al.* 1994a), nitric oxide (Dow, *et al.* 1994b), cGMP and Ca^{2+} (Davies, *et al.* 1995) all stimulate fluid secretion by Malpighian tubules. The utility of this approach is that, by developing a physiological model in *Drosophila*, the unique power of *Drosophila* molecular genetics can be brought to bear on the elucidation and manipulation of the system. This model also has potential *in vivo* manipulation of the NO/cGMP signalling pathway.

Enhancer trapping can not only be used to deduce the formal subdivision of a tissue, depending on the screening of a sufficiently large panel of lines, as has recently been described for *D. melanogaster* mushroom bodies in a collaborative work (Yang *et al.*, 1995), but also can be used to quantify the domains of gene expression to a single cell resolution. Screening of 700 P{GAL4} enhancer trap lines (In collaboration with K. Kaiser's lab) for tubule-specific staining patterns and identification of the most tubule specific lines on the basis of their expression patterns which mark either the tubules, tubule subregions, or tubule subtypes has turned out to have the potential to document previously unsuspected heterogeneity in tubule subregions and cell types specified even to single-cell resolution (Sozen *et al.*, 1996; submitted), as well as providing a route to cloning tubule-specific genes as discussed in **section 4.5**.

The enhancer trap lines suggest that tubules can be divided into initial, transitional, main and lower regions subdivided into three (lower tubule, upper and lower ureter), of which besides non-transporting initial and transitional region only the main region secretes urine, and the lower region reabsorbs it. Additionally, there are at least three cell subtypes, the principal cell (type I), an intercalated, secondary (stellate or type II) cell, and tiny cells of lower region. Classical physiology suggests that cations are pumped through the principal cells (Maddrell and O'Donnell, 1992) while anions flow passively through the stellate cells (Pannabecker, 1993).

There is an additional, and very powerful, use for these lines; as they contain "second generation" P{GAL4} enhancer reporter elements, which encode a yeast transcription factor (GAL4) with no known *Drosophila* homologue, they allow the expression of genetic constructs of choice to be directed in subsets of the epithelial cells, under the control of the yeast UASG promoter (Brand and Perrimon, 1993; Kaiser, 1993). Some of

enhancer trap lines marking specific subsets of the tubules thus allow, for the first time in any animal, to direct expression of constructs very cleanly to almost any subset of epithelial cells.

This technique can be applied, in combination with other biochemical and physiological techniques, to investigate the cell signalling pathways which control fluid secretion in this model epithelium. This model is particularly attractive, because unlike the cell culture models general to this field, it allows the genetic manipulation of components of this pathway in genetically defined cell subtypes within the *intact* tissue in the *living* organism even developmentally since *Drosophila* is well suited to developmental study. This should provide novel insights into the development of competence of signal transduction in maturing epithelia (Davies, S.A. pers. com.).

With the help of enhancer trap lines it is also likely to elucidate which cells contain the components of the NO/cGMP signalling pathway whether specific to certain subregions, or specific to certain cell types in a better and precise way than the traditional genetic approach of the generation of mutations in genes relevant to Malpighian tubule function. This has been done in the case of several subunits of the vacuolar H⁺-ATPases. Genes encoding 5 different subunits of these enzyme complexes have been characterised and lethal alleles generated in three of them (Davies *et al.*, 1995). However, mutations in house-keeping genes can be relatively unsubtle in their impact on the organism. It is clearly desirable to be able to target genetic defects specifically to the cell-type of interest. P{GAL4} enhancer trap lines will allow the directed expression of genetic constructs of choice either to tubules, to defined tubule subregions, or even to defined cell types within the tubules (Kaiser, 1993). This is thus a unique opportunity to study this signalling pathway in particular, and others in general, in the context of an intact organism.

Additionally, it is possible to target inhibition to specific cells (for example, just the secondary cells) rather than bathing the entire tissue in a rather non-specific drug or expression of any gene of choice to specific cell population such as only stellate cells.

The first breakthrough was realised by crossing one of the stellate cell specific lines (C710) with transgenic flies carrying a UASG-5HT1A

receptor construct. This nervous system-specific *Drosophila* receptor is known to act by raising intracellular cAMP (Witz *et al.*, 1990), and when expressed in stellate cells caused a 5HT-sensitive stimulation of fluid secretion (D.C. Kelly, M. Campbell and M.A. Sözen, in preparation). This is exciting, because present models for tubule fluid secretion do not allocate a role to cAMP in the stellate cell (O'Donnell *et al.*, 1996).

A separate advance has been the development of a UAS_G-aequorin construct, that allows direct measurement of intracellular Ca²⁺ in defined populations of cells (Rosay *et al.*, 1996). In tubules, this has shown that leukokinins, a class of invertebrate peptide hormone, act through calcium, as has been argued previously (O'Donnell *et al.*, 1996). More surprising are the findings that CAP_{2b}, another invertebrate neuropeptide active on tubules (Davies *et al.*, 1995) also acts through calcium in direct contradiction of published results (O'Donnell *et al.*, 1996); and that both of these classes of agonist seem to act on principal and stellate cells with different time courses (Davies *et al.*, in preparation).

Taken together, these data can provide a unique insight into the functional organisation of an epithelium. Despite its extraordinary simplicity -a linear array of just 145 cells- these are arranged in a structurally sophisticated manner, and disclose previously unsuspected heterogeneity in both tubule regions and cell types. This complexity of organisation will prove to be a general property of epithelia; however, because of the unique ease with which enhancer trap lines can be generated and screened in *Drosophila*, it is unlikely that similar analyses can be performed on any other epithelial models, except perhaps mouse. Even in the apparently simplest of epithelia, enhancer trapping reveals unexpected complexity in both regions and cell types. However, indirect approaches will ultimately also be of use in such cases; as known genes and proteins are characterised, their distributions can be mapped within a tissue, so building the sophistication of each model.

These results have also presented some evidence and clues about the specialisation of regions and cell type. After the observation of multiple subtypes (mostly not with the same expression patterns) in such a simple tissue in genetically, physiologically and even sometimes morphologically distinct regions, it would be reasonable to conceive a functional specialisation (Meulmans and De Loof, 1992; Maddrell and O'Donnell,

1996) between main cell types (Type I and type II) and regions as in higher eukaryotes (Alper *et al.*, 1989). Work on *D. melanogaster* has frequently pioneered new methodologies, as well as being a model in study of gene structure and function either during development or in mature tissues (Zhao *et al.*, 1995; Dahmane *et al.*, 1995; Campbell *et al.*, 1995; Eccles *et al.*, 1992; Chan *et al.*, 1992; Zhong *et al.*, 1993; Tessarollo *et al.*, 1992; Horan *et al.*, 1995; Smith *et al.*, 1992).

Additionally, the use of cell counting to reconcile boundaries provides a methodology which will allow other enhancer trap analyses to extend beyond the anecdotal, and reveals genetic boundaries to be both precisely placed and functionally significant. As far as is known, this is the first time that enhancer trap analysis has been used in this way to establish cell numbers for expression domains, and to reconcile apparently similar subregions numerically. However, this methodology is in principle applicable to other tissues in both *Drosophila* and in other organisms, as the genetic tools available for the study of vertebrates develop. Its utility in allowing a reconciliation with functional properties should be obvious; and as the *Drosophila* tubule is unlikely to be unique, suggests that epithelia in general may be organised to a level of cellular specialisation which work to date has failed to reveal.

It can be hoped that, by analysis of this simple *Drosophila* model these data may lead to uncover general organising principles as in the developmental analysis of *Drosophila* tubules, which will help to guide such work.

References

- Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J. D. (1994). *Molecular Biology of Cell*. New York, London: Garland Publishing.
- Alper, S. L., Natale, J., Gluck, S., H.F., L. and Brown, D. (1989). Subtypes of intercalated cells in rat kidney collecting duct defined by antibodies against erythroid band 3 and renal vacuolar H⁺-ATPase. *Proc. Natl. Acad. Sci. USA* **86**, 5429-5433.
- Ashburner, M. (1989). *Drosophila: a laboratory manual*: Cold Spring Harbour Laboratory Press.
- Atchison, M. L. (1988). Enhancers: mechanisms of action and cell specificity. *Ann. Rev. Cell Biol.* **4**, 127-153.
- Banerji, J., Olson, L. and Schaffner, W. (1983). A lymphocyte-specific cellular enhancer is located downstream of the joining region in immunoglobulin heavy chain genes. *Cell* **33**, 729-740.
- Bellen, H. J., O'Kane, C., Wilson, C., Grossniklaus, U., Pearson, R. K. and Gehring, W. J. (1989). P-element-mediated enhancer detection: a versatile method to study development in *Drosophila*. *Genes and Development* **3**, 1288-1300.
- Bellen, J. H., Wilson, C. and Gehring, W. J. (1990). Dissecting the complexity of the nervous system by enhancer detection. *BioEssays* **12**, 199-204.
- Bertram, G., Shleithoff, L., Zimmermann, P. and Wessing, A. (1991). Bafilomycin-A1 is a potent inhibitor of urine formation by Malpighian tubules of *Drosophila hydei* - is a vacuolar-type ATPase involved in ion and fluid secretion? *Journal of Insect Physiology* **37**, 201-209.
- Bertram, M. J., Akerkar, G. A., Ard, R. L., Gonzalez, C. and Wolfner, M. F. (1992). Cell type-specific gene expression in the *Drosophila melanogaster* male accessory gland. *Mechanisms of Development* **38**, 33-40.

- Bier, E., Vaessin, H., Shepherd, S., Lee, K., McCall, K., Barbel, S., Ackermann, L., Carretto, R., Uemura, T., Grell, E., Jan, L. Y. and Jan, L. N. (1989). Searching for pattern and mutation in the *Drosophila* genome with a P-lacZ vector. *Genes and Development* **3**, 1273-1287.
- Bingham, P. M., Levis, R. and Rubin, G. M. (1981). The cloning of the DNA sequences from the *white* locus of *Drosophila melanogaster* using a novel and general method. *Cell* **25**, 693-704.
- Blochlinger, K., Bodmer, R., Jack, J., Jan, L. Y. and Jan, Y. N. (1988). Primary structure and expression of a product from *cut*, a locus involved in specifying sensory organ identity in *Drosophila*. *Nature* **333**, 629-635.
- Bradley, T. J. (1984). The excretory system: Structure and Physiology. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 4 (ed. G. A. Kerkut and L. A. Gilbert), pp. 421-466. Oxford/New York/Toronto/Sydney/Paris/Frankfurt: Pergamon Press.
- Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.
- Campbell, C., Goodrich, K., Casey, G. and Beatty, B. (1995). Cloning and mapping of a human gene (*TBX2*) sharing a highly conserved protein motif with the *Drosophila omb* gene. *Genomics* **28**, 255-260.
- Chan, S. D., Karpf, D. B., Fowlkes, M. E., M., H., Bradley, M. S., Vuong, V., Bambino, T., Liu, M. Y., Arnaud, C. D. and Strewler, G. J. (1992). Two homologs of the *Drosophila* polarity gene frizzled (*fz*) are widely expressed in mammalian tissues. *J Biol Chem* **267**, 25202-25207.
- Clancy, C. W. (1955). An anomalous pigmentation of the Malpighian tubules in *Drosophila melanogaster* associated with a variegated-type position effect on the eyes. *Genetics* **40**, 567-568.
- Dahmane, N., Charron, G., Lopes, C., Yaspo, M. L., Maunoury, L., Decorte, P., Sinet, M., Bloch, B. and Delebar, J. M. (1995). Down syndrome-critical region contains a gene homologous to *Drosophila sim* expressed during rat and human central nervous system development. *Proc Natl Acad Sci U S A* **92**, 9191-9195.

Darnell, J. E. (1982). Variety in the level of gene control in eukaryotic cells. *Nature* **297**, 365-371.

Davies, S. A., Huesmann, G. R., Maddrell, S. H. P., O'Donnell, M. J., Dow, J. A. T. and Tublitz, N. J. (1995). CAP_{2b}, a cardioacceleratory peptide, is present in *Drosophila* and stimulates tubule fluid secretion via cGMP. *Am.J. Physiol.* **269**, R1321-R1326.

Davies, S. A., Kelly, D. C., Goodwin, S. F., Yang, S., Sozen, M. A., Kaiser, K. and Dow, J. A. T. (1996). Analysis of *vha55*, the gene encoding the V-ATPase B-subunit in *Drosophila melanogaster* reveals a novel role for proton pumping in transcriptional silencing by *Polycomb*. *J. of Biol. Chem.* **In press**.

Devic, M., Delseny, M. and Gallois, P. (1992). Promoter trapping in *Arabidopsis thaliana* - searching for embryo-specific genes. *Comptes Rendus Des Seances De La Societe De Biologie Et De Ses Filiales* **186**, 541-549.

Doe, C. Q., ChuLaGraff, Q., Wright, D. M. and Scott, M. P. (1991). The prospero gene specifies cell fates in the *Drosophila* central nervous system. *Cell* **65**, 451-464.

Dow, J. A. T. (1994). V-ATPases in insects. In *Organellar proton-ATPases* (ed. N. Nelson), pp. 75-102. Austin, Texas: R.G. Landes Company.

Dow, J. A. T., Goodwin, S. F. and Kaiser, K. (1992). Analysis of the gene encoding a 16-kDa proteolipid subunit of the vacuolar H⁺-ATPase from *Manduca sexta* midgut and tubules. *Gene* **122**, 355-360.

Dow, J. A. T., Kelly, D. C., Davies, S. A., Maddrell, S. H. P. and Brown, D. (1995). A member of the Major Intrinsic Protein family in *Drosophila* tubules. *J. Physiol.* **489**, 110P.

Dow, J. A. T. and Maddrell, S. H. P. (1993). Fluid secretion by the Malpighian tubule of *Drosophila melanogaster* is stimulated by nitric oxide and cyclic GMP. *Journal of Physiology* **473**, 233P.

Dow, J. A. T., Maddrell, S. H. P., Davies, S.-A., Skaer, N. J. V. and Kaiser, K. (1994b). A novel role for the nitric oxide/cyclic GMP

signalling pathway: the control of fluid secretion in *Drosophila*. *American Journal of Physiology* **266**, R1716-R1719.

Dow, J. A. T., Maddrell, S. H. P., Görtz, A., Skaer, N. V., Brogan, S. and Kaiser, K. (1994a). The Malpighian tubules of *Drosophila melanogaster*: a novel phenotype for studies of fluid secretion and its control. *J. exp. Biol.* **197**, 421-428.

Dynan, W. S. and Tjian, R. (1985). Control of eukaryotic mRNA synthesis by sequence specific DNA-binding proteins. *Nature* **316**, 774-778.

Eccles, M. R., Wallis, L. J., Fidler, A. E., Spurr, N. K., Goodfellow, P. J. and Reeve, A. E. (1992). Expression of the PAX2 gene in human fetal kidney and Wilms' tumor. *Cell Growth Differ* **3**, 279-289.

Engels, W. R. (1983). The P family of transposable elements in *Drosophila*. *Ann. Review Genet.* **17**, 315-344.

Engels, W. R. (1989). P elements in *Drosophila melanogaster*. In *Mobile DNA* (ed. D. E. Berg and M. M. Howe), pp. 437-484: American Society for Microbiology.

Engels, W. R. (1992). The origin of P elements in *Drosophila melanogaster*. *BioEssays* **14**, 681-686.

Ferveur, J. F., Stortkuhl, K. F., Stocker, R. F. and Greenspan, R. J. (1995). Genetic feminisation of brain structures and changed sexual orientation in male *Drosophila melanogaster*. *Science* **267**, 902-905.

Finnegan, D. J. (1992). Transposable elements. *Current Opinion in Genetics and Development* **2**, 861-867.

Fischer, J. A., Giniger, E., Maniatis, T. and Ptashne, M. (1988). GAL4 activates transcription in *Drosophila*. *Nature* **332**, 853-856.

Garayoa, M., Villaro, A. C. and Sesma, P. (1994). Myoendocrine-like cells in invertebrates-occurrence of noncardiac striated secretory-like myocytes in the gut of the ant formica- polycтена. *Gen. Comp. Endocrinol.* **95**, 133-142.

Gaul, U., Seifert, E., Schuh, R. and Jackle, H. (1987). Analysis of *Kruppel* protein distribution during early *Drosophila* development reveals posttranslational regulation. *Cell* **50**, 639-647.

Gaul, U. and Weigel, D. (1990). Regulation of *Kruppel* expression in the anlage of the Malpighian tubules in the *Drosophila* embryo. *Mechanisms of Development* **33**, 57-67.

Gillies, S. D., Morrison, S. L., Oi, V. T. and Tonegawa, S. (1983). A tissue-specific transcription enhancer element is located in the major intron of a rearranged immunoglobulin heavy chain gene. *Cell* **33**, 717-728.

Gloor, G. B., Nassif, N. A., Johnson-Schlitz, D. M., Preston, C. R. and Engels, W. E. (1991). Targeted genes replacement in *Drosophila* via P element-induced gap repair. *Science* **253**, 1110-1117.

Gluck, S. and Nelson, R. (1992). The role of the V-ATPase in renal epithelial H⁺ transport. *J.exp.Biol.* **172**, 205-218.

Grawe, F., Wodarz, A., Lee, B., Knust, E. and Skaer, H. (1996). The *Drosophila* genes *crumbs* and *stardust* are involved in the biogenesis of adherens junctions in polarised epithelia. *Development* **122**, 951-959

Greig, S. and Akam, A. (1993). Homeotic genes autonomously specify one aspect of pattern in the *Drosophila* mesoderm. *Nature* **362**, 630-632.

Harbecke, R. and Janning, W. (1989). The segmentation gene *Kruppel* of *Drosophila melanogaster* has homeotic properties. *Genes and Development* **3**, 114-122.

Hartenstein, V. and Jan, Y. N. (1992). Studying *Drosophila* embryogenesis with P-lacZ enhancer trap lines. *Roux's Archives of Developmental Biology* **201**, 194-220.

Hartman-Goldstein, I., Koliantz, G. and Hoyland, M. (1976). Methods for studying the distribution of pigment in Malpighian tubules of *Drosophila melanogaster*. *Stain Technology* **51**, 119-123.

Harvey, B. J. (1992). Energization of sodium-absorption by the H⁺-ATPase pump in mitochondria-rich cells of frog skin. *J. exp. Biol.* **172**, 289-309.

Herr, W. and Clarke, J. (1986). The SV40 enhancer is composed of multiple functional elements that can compensate for one another. *Cell* **45**, 461-470.

Hoch, M., Broadie, K., Jackle, H. and Skaer, H. (1994). Sequential fates in a single cell are established by the neurogenic cascade in the Malpighian tubules of *Drosophila*. *Development* **120**, 3439-3450.

Horan, G. S., Kovacs, E. N., Behringer, R. R. and Featherstone, M. S. (1995). Mutations in paralogous Hox genes result in overlapping homeotic transformations of the axial skeleton: evidence for unique and redundant function. *Dev Biol* **169**, 359-372.

Jan, L. Y. and Jan, Y. N. (1982). Antibodies to horseradish peroxidase as specific neuronal markers in *Drosophila* and grasshopper embryos. *Proc. natn. Acad. Sci. USA*. **79**, 2700-2704.

Jan, Y. N. and Jan, L. Y. (1990). Genes required for specifying cell fates in *Drosophila* embryonic sensory nervous system. *Trends In Neurosciences* **13**, 493-498.

Janning, W., Lutz, A. and Wissen, D. (1986). Clonal analysis of the blastoderm anlage of the Malpighian tubules in *Drosophila melanogaster*. *Roux's Archives of Developmental Biology* **195**, 22-32.

Johnson, P. F. and McKnight, S. L. (1989). Eukaryotic transcriptional regulatory proteins. *Annual Review of Biochemistry* **58**, 797-839.

Kaiser, K. (1993). Transgenic *Drosophila* - 2nd-generation enhancer traps. *Current Biology* **3**, 560-562.

Kersting, U., Napathorn, S. and Spring, K. R. (1993). Necturus gallbladder epithelial cell volume regulation and inhibitors of arachidonic acid metabolism. *J. of Membrane Biology* **135**, 11-18.

Klein, U. (1992). The insect V-ATPase, a plasma-membrane proton pump energizing secondary active transport - immunological evidence for the occurrence of a V-ATPase in insect ion-transporting epithelia. *J.exp.Biol.* **172**, 345-354.

- Knowles, G. (1975). The reduced glucose permeability of the isolated Malpighian tubules of the blowfly *Calliphora vomitoria*. . *J.exp.Biol.* **62**, 327-340.
- Kurosaka, A., Yano, A., Itoh, N., Kuroda, Y., Nakagawa, T. and Kawasaki, T. (1991). The structure of a neural specific carbohydrate epitope of horseradish peroxidase recognized by anti-horseradish peroxidase antiserum. *J. Biol. Chem.* **266**, 4168--4172.
- Laski, F. A., Rio, D. C. and Rubin, G. M. (1986). Tissue specifity of *Drosophila* P element transposition is regulated at the level of mRNA splicing. *Cell* **44**, 7-19.
- Lawrence, P. A. (1993). The making of a fly. Oxford: Blackwell Scientific.
- Lewin, B. (1994). Genes V. Oxford: Oxford University Press.
- Lindsey, D. L. and Zimm, G. G. (1992). The genome of *Drosophila melanogaster*. New York: Academic Press.
- Lindsey, K., Wei, W. B., Clarke, M. C., Mcardle, H. F., Rooke, L. M. and Topping, J. F. (1993). Tagging genomic sequences that direct transgene expression by activation of a promoter trap in plants. *Transgenic Research* **2**, 33-47.
- Liu, S. and Jack, J. (1992). Regulatory interactions and role in cell type specification of the Malpighian tubules by the *cut*, *Kruppel*, and *caudal* genes of *Drosophila*. *Developmental Biology* **150**, 133-143.
- Liu, S., Mcleod, E. and Jack, J. (1991). Four distinct regulatory regions of the *cut* locus and their effect on cell type specification in *Drosophila*. *Genetics* **127**, 151-159.
- Macdonald, P. M. and Struhl, G. (1986). A molecular gradient in early *Drosophila* embryos and its role in specifying the body pattern. *Nature* **324**, 537-545.
- Maddrell, S. H. P., Lane, N.J., Harrison, J.B., Gardiner, B.O.C. (1985). DNA replication in binucleate cells of the Malpighian tubules of Hemipteran insects. *Chromosoma* **91**, 201-209.

- Maddrell, S. H. P. (1991). The fastest fluid-secreting cell known: the upper Malpighian tubule cell of *Rhodnius*. *BioEssays* **13**, 357-362.
- Maddrell, S. H. P., Gardiner, B. O. C., Pilcher, D. E. M. and Reynolds, S. E. (1974). Active transport by insect Malpighian tubules of acidic dyes and of acylamides. *Journal of Experimental Biology* **61**, 357-377.
- Maddrell, S. H. P., Herman, W. S., Farndale, R. W. and Riegel, J. A. (1993). Synergism of hormones controlling epithelial fluid transport in an insect. *Journal of Experimental Biology* **174**, 65-80.
- Maddrell, S. H. P., Herman, W. S., Mooney, R. L. and Overton, J. A. (1991). 5-hydroxytryptamine: a second diuretic hormone in *Rhodnius prolixus*. *Journal of Experimental Biology* **156**, 557-566.
- Maddrell, S. H. P. and O'Donnell, M. J. (1992). Insect Malpighian tubules: V-ATPase action in ion and fluid transport. *Journal of Experimental Biology* **172**, 417-429.
- Maniatis, T., Goodbourn, J. and Fischer, J. A. (1987). Regulation of Inducible and tissue-specific gene expression. *Science* **236**, 1237-1244.
- Meulemans, W. and De Loof, A. (1992). Transport of the cationic fluorochrome rhodamine 123 in an insect's Malpighian tubule: Indications of a reabsorptive function of the secondary cell type. *Journal of Cell Science* **101**, 349-361.
- Mlodzik, M., Fjose, A. and Gehring, W. J. (1985). Isolation of caudal, a *Drosophila* homeobox-containing gene with maternal expression, whose transcripts form a concentration gradient at the pre-blastoderm stage. *EMBO J.* **4**, 2961-2969.
- Moffat, K. G., Gould, J. H., Smith, H. K. and O'Kane, C. J. (1992). Inducible cell ablation in *Drosophila* by cold-sensitive ricin-A chain. *Development* **114**, 681.
- Nose, A., Mahajan, V. B. and Goodman, C. S. (1992). Connectin: A homophilic cell adhesion molecule expressed on a subset of muscles and the motoneurons that innervate them in *Drosophila*. *Cell* **70**, 553-567.

- O'Dell, K. M. C., Armstrong, J. D., Yang, M. Y. and Kaiser, K. (1995). Functional dissection of the *Drosophila* mushroom bodies by selective feminization of genetically defined subcompartments. *Neuron* **15**, 55-61.
- O'Donnell, M. J., Dow, J. A. T., Huesmann, G. R., Tublitz, N. J. and Maddrell, S. H. P. (1996). Separate control of anion and cation transport in Malpighian tubules of *Drosophila melanogaster*. *Journal of Experimental Biology* **199**, 1163-1175.
- O'Donnell, M. J. and Maddrell, S. H. P. (1995). Fluid reabsorption and ion transport by the lower Malpighian tubules of adult female *Drosophila*. *Journal of Experimental Biology* **198**, 1647-1653.
- O'Kane, C. J. and Gehring, W. J. (1987). Detection *in situ* of genomic regulatory elements in *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 9123-9127.
- O'Kane, C. J. and Moffat, K. G. (1992). Selective cell ablation and genetic surgery. *Current Opinion in Genetics and Development* **2**, 602-607.
- Pannabecker, T. L., Hayes, T. K. and Beyenbach, K. W. (1993). Regulation of epithelial shunt conductance by the peptide leucokinin. *Journal of Membrane Biology* **132**, 63-76.
- Parker, S. C. and Topol, J. (1984a). A *Drosophila* RNA polymerase II transcription factor binds to the regulatory site of an hsp 70 gene. *Cell* **37**, 273-283.
- Parker, S. C. and Topol, J. (1984b). A *Drosophila* RNA polymerase II transcription factor contains a promoter-region-specific DNA-binding activity. *Cell* **36**, 357-369.
- Pelham, H. (1985). Activation of heat-shock genes in eukaryotes. *Trends in Genetics* **1**, 31-34.
- Riesgo-Escovar, J., Woodard, C., Gaines, P. and Carlson, J. (1992). Development and organization of the *Drosophila melanogaster* olfactory system: An analysis using enhancer traps. *Journal of Neurobiology* **23**, 947-964.

- Rio, D. C. (1991). Regulation of *Drosophila* P element transposition. *Trends in Genetics* **7**, 282-287.
- Robertson, H. M., Preston, C. R., Phillis, R. W., Johnson-Schlitz, D. M., Benz, W. K. and Engels, W. R. (1988). A stable source of P element transposase in *Drosophila melanogaster*. *Genetics* **118**, 461-470.
- Rosay, P., Davies, S. A., Sozen, M. A., Dow, J. A. T. and Kaiser, K. (1996). A cell-type specific system for monitoring calcium in *Drosophila* using an aequorin expression system. *Eur. J. Neuroscience* (in press), (abstract).
- Rosenberg, U., Schroder, C., Preiss, A., Kienlin, A., Cote, S., Riede, I. and Jackle, H. (1986). Structural homology of the product of the *Drosophila* Kruppel gene with *Xenopus* transcription factor IIIA. *Nature* **319**, 336-339.
- Rothstein, A. (1989). The Na⁺/H⁺ exchange system in cell pH and volume control. *Reviews of physiology, biochemistry and pharmacology* **112**, 235-257.
- Rubin, G. M. (1988). *Drosophila melanogaster* as an experimental organism. *Science* **240**, 1453-1459.
- Rubin, G. M. and Spradling, A. C. (1982). Genetic transformation of *Drosophila* with transposable element vectors. *Science* **218**, 348-353.
- Russell, V. E. W., Klein, U., Reuveni, M., Spaeth, D. D., Wolfersberger, M. G. and Harvey, W. R. (1992). Antibodies to mammalian and plant V-ATPases cross react with the V-ATPase of insect cation-transporting plasma membranes. *J.exp.Biol.* **166**, 131-143.
- Salz, H. K., Cline, T. W. and Schedl, P. (1987). Functional changes associated with structural alterations induced by mobilization of a P element inserted in the *sex-lethal* gene of *Drosophila*. *Genetics* **117**, 221-231.
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular cloning: a laboratory manual: Cold Spring Harbor Laboratory Press.
- Sanger, F., Nicklen, S. and Coulson, A. R. (1977). DNA sequencing with chain terminating inhibitors. *Proc. Nat. Acad. Sci. USA* **74**, 5463-5467.

- Schubiger, M., Feng, Y., Fambrough, D. M. and Palka, J. (1994). A mutation of the *Drosophila* sodium pump alpha subunit gene results in bang- sensitive paralysis. *Neuron* **12**, 373-381.
- Searles, L. L., Jokerst, R. S., Bingham, P. M., Voelker, R. A. and Greenleaf, A. L. (1982). Molecular cloning of sequences from a *Drosophila* RNA polymerase II locus by P element transposon tagging. *Cell* **31**, 585-592.
- Sentry, J. W. and Kaiser, K. (1992). P-element transposition and targeted manipulation of the *Drosophila* genome. *Trends In Genetics* **8**, 329-331.
- Sentry, J. W., Yang, M. M. and Kaiser, K. (1993). Conditional cell ablation in *Drosophila*. *Bioessays* **15**, 491-493.
- Serfling, E., Jasin, M. and Schaffner, W. (1985). Enhancers and eukaryotic gene transcription. *Trends In Genetics* **1**, 224-230.
- Siddiqui, S. and Culotti, J. (1984). Invertebrate neurons share a common antigenic determinant recognized by antibodies to horseradish peroxidase. *J. Cell. Biochem.* **8**, 108.
- Skaer, H. (1989). Cell division in Malpighian tubule development in *D. melanogaster* is regulated by a single tip cell. *Nature* **342**, 566-569.
- Skaer, H. (1993). The alimentary canal. In *The Development of Drosophila melanogaster*, vol. 2 (ed. M. Bate and A. Martinez Arias), pp. 941-1012. Cold Spring Harbor: Cold Spring Harbor Press.
- Skaer, H. and Martinez-Arias, A. (1992). The *wingless* product is required for cell proliferation in the Malpighian tubule anlage of *Drosophila melanogaster*. *Development* **116**, 745-754.
- Smith, D., Wohlgemuth, J., Calvi, B. R., Franklin, I. and Gelbart, W. M. (1993). *hobo* Enhancer trapping mutagenesis in *Drosophila* reveals an insertion specificity different from P elements. *Genetics* **135**, 1063-1076.
- Smith, D. E., Franco del Amo, F. and Gridley, T. (1992). Isolation of *Sna*, a mouse gene homologous to the *Drosophila* genes *snail* and *escargot*: its expression pattern suggest multiple roles during postimplantation development. *Development* **116**, 1033-1039.

- Smith, H. K. and O'Kane, C. J. (1991). Use of a cytoplasmically localised P-lacZ fusion to identify cell shapes by enhancer trapping in *Drosophila*. *Roux's Archives of Developmental Biology* **200**, 306-311.
- Soininen, R., Schoor, M., Henseling, U., Tepe, C., Kisters-Woike, B., Rossant, J. and Gossler, A. (1992). The mouse Enhancer trap locus 1 (Etl-1): A novel mammalian gene related to *Drosophila* and yeast transcriptional regulator genes. *Mechanisms of Development* **39**, 111-123.
- Sozen, M. A., Armstrong, J. D., Yang, M.-Y., Kaiser, K. and Dow, J. A. T. (1996). Functional compartments are specified to single-cell resolution in a *Drosophila* epithelium. **Submitted**.
- Spradling, A. C. and Rubin, G. M. (1982). Transposition of cloned P elements into *Drosophila* germ line chromosomes. *Science* **218**, 341-347.
- Struhl, K. (1987). Promoters, activator proteins, and the mechanism of transcriptional initiation in yeast. *Cell* **49**, 295-297.
- Tepass, U. and Knust, E. (1990). Phenotypic and developmental analysis of mutations at the *crumbs* locus, a gene required for the development of epithelia in *Drosophila melanogaster*. *Roux's Archives of Developmental Biology* **199**, 189-206.
- Tessarollo, L., Nagarajan, L. and Parada, L. F. (1992). c-ros: the vertebrate homolog of the sevenless tyrosine kinase receptor is tightly regulated during organogenesis in mouse embryonic development. *Development* **115**, 11-20.
- Topping, J. F., Agyeman, F., Henricot, B. and Lindsey, K. (1994). Identification of molecular markers of embryogenesis in *Arabidopsis thaliana* by promoter trapping. *Plant Journal* **5**, 895-903.
- Tower, J., Karpen, J. H., Craig, N. and Spradling, A. C. (1993). Preferential transposition of *Drosophila* P elements to nearby chromosomal sites. *Genetics* **133**, 347-359.
- Tsubota, S. and Schedl, P. (1986). Hybrid dysgenesis-induced revertants of insertions at the 5' end of the *rudimentary* gene in *Drosophila melanogaster*: transposon-induced control mutations. *Genetics* **114**, 165-182.

Van Breugel, F. M. A. (1973). Cell clustering and pleiotropy in white-variegated eyes and Malpighian tubes of *Drosophila hydei*. *Genetics* **75**, 323-334.

Verbavatz, J. M., Van Hoek, A. N., Ma, T., Sabolic, I., Valenti, G., Ellisman, M. H., Ausiello, D. A., Verkman, A. S. and Brown, D. (1994). A 28 kDa sarcolemmal antigen in kidney principal cell basolateral membranes: Relationship to orthogonal arrays and MIP 26. *Journal of Cell Science* **107**, 1083-1094.

Watson, J. D., Gilman, M., Witkowski, J. and Zoller, M. (1992). Recombinant DNA. New York: Scientific American.

Weigel, D., Jurgens, G., Kuttner, F., Seifert, E. and Jackle, H. (1989). The homeotic gene fork head encodes a nuclear protein and is expressed in the terminal regions of *Drosophila* embryo. *Cell* **57**, 645-658.

Wessing, A. and Eichelberg, D. (1978). Malpighian tubules, rectal papillae and excretion. In *The genetics and biology of Drosophila*, vol. 2c (ed. A. Ashburner and T. R. F. Wright), pp. 1-42. London: Academic Press.

Wilkins, A. S. (1995). Singling out the tip cell of the Malpighian tubules-lessons from neurogenesis. *BioEssays* **17**, 199-202.

Wilson, C., Pearson, P. K., Bellen, H. J., O'Kane, C. J., Grossniklaus, U. and Gehring, W. J. (1989). P-element-mediated enhancer detection: Isolation and characterization of developmentally regulated genes in *Drosophila*. *Genes and Development* **3**, 1301-1313.

Witz, P., Amlaiky, N., Plassat, J. L., Maroteaux, L., E., B. and Hen, R. (1990). Cloning and characterization of a *Drosophila* serotonin receptor that activates adenylate cyclase. *Proc. Natl. Acad. Sci. USA* **87**, 8940-8944.

Yang, M. Y., Armstrong, J. D., Vilinsky, I., Strausfeld, N. J. and Kaiser, K. (1995). Subdivision of the *Drosophila* mushroom bodies by enhancer-trap expression patterns. *Neuron* **15**, 45-54.

Zhang, P. and Spradling, A. C. (1993). Efficient and dispersed local P-element transposition from *Drosophila* females. *Genetics* **133**, 361-373.

Zhao, Z., Lee, C. C., Baldini, A. and Caskey, C. T. (1995). A human homologue of the *Drosophila* polarity gene *frizzled* has been identified and mapped to 17q21.1. *Genomics* **27**, 370-373.

Zhong, W., Sladek, F. M. and Darnell, J. E. (1993). The expression pattern of a *Drosophila* homolog to the mouse transcription factor HNF-4 suggest a determinative role in gut formation. *EMBO J* **12**, 537-544.

Zierold, K. and Wessing, A. (1990). Mass dense vacuoles in *Drosophila* Malpighian tubules contain zinc, not sodium. A reinvestigation by X-ray microanalysis of cryosections. *European Journal of Cell Biology* **53**, 222-226.